DETECTION OF NS1 ANTIGEN/IgM, IgG ANTIBODIES IN EARLY DENGUE VIRUS INFECTION AMONG THE PATIENTS ATTENDING SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES, KULASEKHARAM.

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CERTIFICATE

This is to certify that the dissertation entitled **"DETECTION OF NS1 ANTIGEN/IgM, IgG ANTIBODIES IN EARLY DENGUE VIRUS INFECTION AMONG THE PATIENTS ATTENDING SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES, KULASEKHARAM"** is a bonafide work done by **DR.VIDHYA V.R**. from **SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES, KULASEKHARAM** in partial fulfillment of the University rules and regulations for award of degree of **Doctor of Medicine in Microbiology (Branch-1V)** of Tamil Nadu Dr. M.G.R. Medical University.

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The dissertation is submitted to the Tamil Nadu Dr MGR Medical University, towards the partial fulfilment of requirements for the award of M.D degree (Branch - IV) in Microbiology.

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CONTENTS

DETECTION OF NS1 ANTIGEN /IgM, IgG ANTIBODIES IN EARLY DENGUE VIRAL INFECTION AMONG THE PATIENTS ATTENDING SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES , KULASEKHARAM.

ABSTRACT

Introduction;

Dengue has become a major international public health problem due to human morbidity and mortality it causes. The disease presents as acute febrile illness with chills, headache, retro-oular pain, body aches and arthralgia in more than 90% of apparent cases accompanied by nausea, vomiting and a maculopapular rash resembling measles. . Aedes aegyptyi is the primary vector mosquito. Dengue virus is a positive stranded encapsulated, RNA virus. . Dengue NS1 antigen has been detected in the serum of DEN virus infected patients as early as one day of onset of symptoms. In this study the potential use of dengue NS1 antigen and IgM and IgG antibodies in early dengue diagnosis has been analysed.

Materials and methods;

The study group consists of all febrile patients with fever of duration less than 5 days attending the medical OPD for a period of one year from July 2013. A totalof 176 blood samples from fever patients were received in the Microbiology lab in our instituition . Each sample was subjected to an immunochromatographic test (card test) for NS1 antigen , IgM and IgG antibodies . This was followed by ELISA for NS1 antigen, IgM and IgG antibodies. The other parameters such as age , duration of fever, gender and platelet count were also recorded.

Results;

Out of 176 samples tested , 57 (32%) were positive only for NS1 antigen, 7 (4%) samples were found to be positive for IgM antibodies and none was found to be positive for IgG antibodies by immunochromatographic test . The 57 samples which were found to be positive by NS1 ELISA were also found to be positive by immunochromatographic test. By ELISA method 37 samples (21%) were positive for IgM and 7 samples (4%) were found to be positive for IgG . Out 57 cases positive for NS1 antigen ,thrombocytopenia was evident in 42 cases (74%) .In antibody positive cases thrombocytopenia was noted in 20 out of 44 cases (45%).

Conclusion;

NS1 antigen detection is a useful tool in the early diagnosis of dengue infection and deserves inclusion in the diagnostic algorithm for suspected dengue fever cases .The results of NS1 antigen ELISA is comparable to that of NS1 antigen ELISA .But in case of antibodies the immunochromatographic test is inferior to that of ELISA.

Key words : Dengue, NS1 antigen, IgM &IgG antibodies, platelet, immunochromatographic test, ELISA,Early detection of dengue virus.

INTRODUCTION

Dengue virus which is found mainly in the tropical and subtropical regions worldwide belongs to family Flaviviridae . Dengue fever is an acute, potentially fatal viral infection that is endemic throughout India[1]. According to the Oxford English Dictionary, the term dengue originated in the Swahili phrase " ka dinga pepo " (a kind of sudden cramp like seizures from an evil spirit or plague). Dr. James Cahristies (1872) proposed that the disease crossed from East Africa to the Caribbean in1827[2].

The dengue virus originated in the forests, evolving from the mosquito virus to one capable of causing disease in humans. Deforestation by humans had helped the dengue virus to move out of the out of the jungle to the rural environment[3]. As a result of economic development, people began to move out of rural areas to cities which also introduced dengue virus into a new environment. Transmission of dengue virus in Asia is maintained by a native mosquito species , the Aedes albopticus. When the virus was introduced into an area that had become infested with the Aedes aegypti mosquito , dengue epidemics occurred . Now there is evidence that both dengue virus and its primary vector, the Aedes aegypti mosquito had their origins in either Asia or Africa[4].

The first recorded case of dengue fever was documented from a Chinese medical encyclopaedia from the Jin Dynasty (265 -420 AD). The Chinese referred to a water poison associated with flying insects. The first definitive case was reported in 1789 and was attributed to Benjamin Rush, who also coined the term break bone fever

for dengue fever because of the symptoms of muscle and joint pain .The viral etiology and the transmission by the mosquitoes was deciphered only by 20th century. A pandemic of dengue infections began in Southeast Asia after the end of World War II and then it spreaded around the globe[2].

It is seen that global prevalence of Dengue infections has grown dramatically in recent years. According to WHO estimates dengue viral infections are responsible for hundred million cases of dengue infections annually and more than five lakh cases of dengue haemorrhagic fever[5]. The main factors which had made dengue infections a global health problem is unprecedented global population growth, unplanned and uncontrolled urbanisation and deterioration in water, sewer and waste management systems[4]. The emerging dengue viral infections is of great threat to the mankind as there is no specific medication available nor there is any vaccine available against dengue virus.[6]

Dengue virus consists of four distinct serotypes based on their antigens, namely DENV1, DENV2, DENV3 and DENV4. All the four serotypes of Dengue virus (which are closely related) is distinguished by both serology and molecular diagnostic methods. Currently, five subtypes have been identified for DEN-1, six subtypes for DEN-2, four subtypes for DEN-3 and two subtypes for DEN-4. Dengue virus consists of a positive stranded, encapsulated RNA as their genetic material which is 11kb long and has a single open reading frame (ORF) which codes for a single polypeptide. This polypeptide consists of three structural proteins and seven non-structural proteins. The structural proteins are named as core protein C, membrane protein M and envelope protein E. The seven non structural proteins are NS1, NS2A , NS2B , NS3 , NS4A, NS4B and NS5 .[7]

NS1 protein is a 50 kilodalton glycoprotein (353 or 354 amino acids), which is expressed on the surface of cells infected with dengue virus. It is produced in both membrane associated and secreted forms [8]. Several studies conducted revealed that NS1 can be detected in the blood of dengue virus infected patients even before the detection of antibodies[9]. It may be detected in the blood from the first day of fever up to the fifth day of fever which is the critical period of illness preceding defervescence which generally occurs by the fifth day[10,11]. Therefore commercial diagnostic kits have utilised virus coded NS1 antigen as a basis for early detection of dengue viral infections[12]. Currently, NS1 antigen captures ELISA and rapid immunochromatographic tests are available for detection of NS1 antigen. Detection of Dengue NS1 antigen presents a better method for the early diagnosis of dengue viral infections[1]. A positive NS1 antigen test may confirm a dengue infection. The antibodies for dengue infections remains undetectable for months and a positive result obtained on a single blood specimen cannot rule out dengue viral infection[5].

The antibody production following Dengue viral infection consists of IgM and IgG antibodies which are produced against the viral envelope proteins. The type of antibody production may depend on whether an individual has a primary dengue viral infection or a secondary dengue viral infection[11]. In primary dengue viral infection the individual may be exposed to dengue or other flavivirus infection for the first time where as in secondary infection the individual had dengue or any other flavivirus infections in the past[12]. In general, dengue diagnosis depends on the infection phase. A primary dengue viral infection is characterised by a low titre antibody production. The first immunoglobulin isotype to appear in primary dengue infection is IgM . IgM antibodies may be detectable approximately 3 to 5 days after the onset of fever and

then its levels increases rapidly and peaks for 2 weeks after the onset of fever. However IgG immunoglobulins are detectable at low titres by the end first week of illness and then it rises slowly. The level of IgG antibodies decline slowly over the next 3 to 6 months[8].

Dengue virus is transmitted through human – mosquito cycle by Aedes aegypti mosquito. The extrinsic incubation period is 8 – 10 days[13]. Dengue fever has an incubation period of 5-11 days after which the patient presents with fever of sudden onset with headache, retrobulbar pain, conjunctival injection, pain in back and limbs (break- bone fever), lymphadenopathy and maculopapular rash. The fever is typically biphasic (saddle – back) and it may last for five to seven days[14].

Dengue infections can also occur in serious forms with haemorrhagic symptoms known as dengue haemorrhagic fever or with shock known as dengue shock syndrome[2]. This may be due to hypersensitivity reaction to sequential dengue virus infections in persons sensitised by prior exposure to other serotypes of virus .High levels of early NS1 antigens in the blood may be associated with more severe clinical manifestations[15].

The Aedes mosquito bites mainly during the day time with increased biting activity two hours after the sunrise and several hours before sunset, and it feeds itself in the dark corners of the houses or on hanging objects like clothes, umbrella or under the furniture[16]. Aedes aegypti mosquito can breed in any type of containers having very small quantity of water. Eggs of Aedes aegypti can survive without water for almost a year. The favourite breeding habitats are coolers, buckets, cups, broken pots, flower vases, plant saucers , tanks, cisterns, bottles, tins, tyres, coconut shells, tree

holes and many other places where rain water can be collected or stored . So vector control is the most efficient way of controlling dengue fever[17]. The best way to control mosquitoes is to control of mosquito egg laying site by simple measures like adding drainage holes to structures and containers that may trap water (barrels, old tyre). Thin out weeds and remove old leaves from pond , this will allow natural mosquito eating fish to access areas where mosquitoes lay their eggs .The use of pesticides should only be supplemental to controlling mosquitoes through the reduction and management of mosquito egg laying areas [18]. Bacillus thuringiensis serotype H-14 and Bacillus sphaericus can be used as effective mosquito control agents[19]. Larvivorus fish (Gambusia affinis and Poecilia reticulate) can also be introduced for the control of Aedes mosquitoes in large water containers .Another method for protection against mosquito bite is personal protection by wearing long pants and long sleeves to cover the skin. Repellents with DEET (N, N – dimethyl – Meta-toluamide) are the most effective[18]. A well documented phenomenon reported from many parts of the dengue endemic area is transovarial transmission of dengue virus in aedes mosquito, which further emphasized the importance of the larval control since the larval stages has become the reservoir of the dengue virus in the inter-epidemic period.[20]

In this study, the potential use of dengue NS1 antigen in early dengue diagnosis is analysed and compared to the current antibody methods available in our laboratory.

DENGUE CASE DEFINITIONS[21, 22];

[2009 NEW DENGUE CASE DEFINITIONS]

Dengue without any warning signs ;

Fever and any two of the following:

1) Nausea ,vomiting
2) Rash on the body
3) BodyAches and joint pains
4) Leukopenia
5) Positive tourniquet test.

Dengue with warning signs

Dengue as defined above with any of the following :

1) Pain in the abdomen
2) Persistent vomiting
3) Fluid accumulation anywhere in the body (ascites, pleural effusion)
4) Bleeding from the mucosa
5) Weakness, restlessness.
6) Liver enlargement more than 2 cm.

Laboratory: increase in HCT concurrent with rapid decrease in platelet count

**Requires strict observation and medical care.

SEVERE DENGUE;

Dengue with at least one of the following criteria :

- Severe Plasma Leakage leading to:
  - Shock (Dengue Shock Syndrome).
  - Fluid accumulation anywhere in the body with respiratory distress.
- Severe Bleeding as evaluated by clinician
- Severe organ involvement
  - Liver: AST or ALT more than equal to1000.
  - Central nervous system: altered consciousness.
  - Failure of heart and other organs.

The real WHO classification is defined as follow: Dengue Fever (DF), Dengue Haemorrhagic Fever (DHF), and Dengue Shock Syndrome (DSS).

1997 DENGUE CASE DEFINITIONS;

DENGUE FEVER :

Dengue fever is most commonly an acute febrile illness defined by the presence of fever and two or more of the following, retro-orbital or ocular pain, headache, rash, muscle pain, joint pain, leucopoenia, or haemorrhagic manifestations (eg, positive tourniquet test, petechiae, purpura/ecchymosis, bleeding from the gums or nose, blood in vomitus , urine or stool or vaginal bleeding) but not meeting the case definition of dengue haemorrhagic fever. Anorexia, nausea, abdominal pain , and persistent vomiting may also occur but are not case defining criteria for dengue fever.

DENGUE HAEMORRHAGIC FEVER

- Fever lasting from 2—7 days.
- Evidence of haemorrhagic manifestation or a positive tourniquet test.
- Thrombocytopenia (< /=100,000 cells /mm^3)
- Evidence of plasma leakage as shown by haemoconcenteration (an increase in haematocrit >/=20% above average for age or a decrease in haematocrit >/=20 % of baseline following fluid replacement therapy), or pleural effusion, or ascites or hypoproteinemia.

DENGUE SHOCK SYNDROME

DSS has all the criteria of Dengue haemorrhagic fever plus circulatory failure as evidenced by;

- Rapid and weak pulse and narrow pulse pressure (<20 mm Hg) , or
- Age –specific hypotension and cold , clammy skin and restlessness .

EPIDEMIOLOGY OF DENGUE INFECTIONS

The Centre for Disease control has been the primary source of information about the world wide distribution of dengue viral infection .Global incidence of dengue infections during 2010 is rising at the highest rate since 1988. Dengue viral infections have been epidemic in tropical and sub-tropical areas which were previously considered as non-endemic to dengue virus. The main areas are Asia, Americas, and the Caribbean . Typically transmission of dengue virus peaks near the equator during the annual rainy season. There are reports that epidemics for dengue in India and Southeast Asia (like, Indonesia, Sri Lanka, Taiwan, and Thailand) started up

to 4 weeks earlier than normal time, which is mainly due to heavy and prolonged rainfalls. .Among the Central and South American countries, epidemics have been declared in Peru, Venezuela, Colombia, Brazil, Nicaragua, Honduras, and Paraguay. The Dominican Republic has been declared a state of emergency because of alarmingly high case fatalities from dengue fever. A number of areas where dengue has been absent or rare during the 21st century have reported cases, including Australia, Nepal, Jeddah, Saudi Arabia, Reunion Island, Comoros, Tanzania and Senegal.

Since India is endemic to dengue virus, it is very important to study the epidemiology of dengue in India. The first major dengue epidemic occurred in Chennai in 1780's which later spread all over the country [23]. Dengue virus was first isolated in Japan in 1943 but the one which was isolated from Calcutta in 1944 from the blood of the US soldiers had been considered as the first report for dengue virus infection for a long time. When the first epidemic of Dengue haemorrhagic fever occurred during 1996 in Northern India, nothing was known about its emergence. Dengue type 2 viruses were responsible for 1996 epidemics, while the 2003 epidemics were mainly due to dengue type 3 viruses. All the four serotypes of dengue virus have been isolated from India. The first major outbreak of dengue fever in India was reported from Calcutta in 1964[24] followed by an epidemic in Vishakhapatnam in 1965 [25]. Delhi had outbreaks of dengue viral infections due to different dengue serotypes in 1967, 1970, 1982, and 1988.

Isolates from these different (geographically distant) epidemics are closely related and belong to DV-3 subtype III, which initially originated in the India. The emergence of Dengue haemorrhagic fever in Sri Lanka in 1989 coincided with the

appearance of a new dengue virus3, subtype III variant. This variant spreaded from the Indian subcontinent to Africa in 1980 and fro Africa to Latin America in the mid 1990. DV3 subtype III isolates from the mild and severe disease outbreaks form genetically distinct groups which suggested a role of viral genetics in Dengue haemorrhagic fever.[26]

Cases of DHF were reported for the first time in 1988[27]. Although several cases of dengue viral infections occurred in India, only occasional cases of DHF/DSS have been reported. This is rather puzzling because DHF has been very common in South East Asia but rare in the India despite presence of all four serotypes in both regions . Epidemiological studies have shown that the rate of the virus transmission and the amount of each dengue serotypes, remained constant before the emergence of dengue haemorrhagic fevers. The incidence of DHF from 1989 in the Indian sub continent was correlated with the introduction of a new subtype DEN -3 subtype III. It is the predominant one which is genetically distinct from DEN3 previously isolated from the infected persons presenting with severe disease and are inherently more virulent[28].

After 1989, frequent epidemics of DHF occurred in several countries of the Indian subcontinent. DF and DHF have been reported from Coimbatore, Erode in 1998[29]. Another outbreak occurred in Mangalore in 1993[30]. After a gap of eight years a large outbreak of dengue fever of serious nature occurred in Delhi again in 1996. The cause was confirmed as dengue virus type 2 subtype III by virus cultivation and direct immunofluorescence with monoclonal antibodies[31]. During the outbreak from August to November, a total of 10,252 cases and 423 deaths have been reported to

the State Directorate. Delhi, Haryana, Rajasthan, Karnataka and Tamil Nadu have reported dengue in small numbers in 1998 , 1999 , 2000[32].

The major dengue outbreak in North India caused by dengue virus type3 subtype III occurred in 2003 and 2004 . The re-emergence of fatal subtype III of DEN -3 which replaced the earlier circulating subtype 1V of DEN-2 in India is a matter of concern[33]. An outbreak of Dengue haemorrhagic fever and dengue shock syndrome occurred in Delhi in 2006. This is the first report from India with high percentage of concurrent infections with different dengue virus serotypes circulating during one outbreak [34].

Epidemiology of Dengue in Tamil Nadu;

Statistics from the National Vector Borne Disease Control Programme (NVBDCP) show that the state recorded 677 cases after Maharashtra (963) and was followed by Kerala (651). According to experts, in the state's rural area , acute water shortages forces people to store water in vessels that help breed mosquitoes. Dengue virus serotypes 2 and 3 are mainly found in this regions.[35]

PATHOGENESIS OF DENGUE FEVER

After an infectious mosquito bite,virus which replicates in local lymph nodes and disseminates via blood to various tissues within the next 2-3 days. Virus circulates in infected monocytes and macrophages and to a lesser degree in B and T cells for 5 days. It also replicates in skin, reactive spleen, lymphoid cells and macrophages.

In liver viral antigen can be demonstrated in Kuppfer cells and endothelia. Changes occurring in liver include hypertrophy of kupffer cells, focal ballooning and

necrosis of hepatocytes with occasional Councilman Body formation. Skin rashes comprise lymphocytic dermal vasculitis and viral antigen. Malaise and flu like symptoms reflect cytokine response. Myalgia is due to moderate perivascular mononuclear infiltrate with lipid accumulation[37].

Central nervous system abnormalities can be attributed to metabolic alterations, haemorrhagic manifestations, viral CNS invasion and encephalitis. Shock ensues when there are sudden extravasations of plasma into the extra vascular sites including pleural and abdominal cavities usually with defervescence of fever. Dengue virus NS1 protein or the antibodies to dengue infection might interact with glycocalyx layer of endothelium in such a way as to alter temporarily the characteristics of the fibre matrix which may contribute to the increased permeability of the endothelium . Enhanced fibrinolytic activity develops due to the interaction between viral particle and plasminogen. Release of Heparin sulphate or Chondroitin sulphate from endothelial membrane also contributes to the mechanism. Procoagulant markers are increased to some degree with a decrease in anticoagulant proteins[38].

Dominant epitopes that react with antigen responsive T lymphocytes are peptides of the dengue non structural protein NS3. During secondary infections expansion of lower avidity memory T cells takes precedence over that of the naive T cells with higher avidity for the new DEN-5 serotype. CD8+T cells generated during infection binds weakly to the MHC tetramers presenting epitopes of the infecting virus. A frequency of these cells shows an apoptotic phenotype and seems destined to die before the infection is adequateycontrolled. Low avidity T cells that dominate the response to secondary infection are less optimally efficient at elimination of dengue virus infected cells . Infants in endemic areas fail to develop dengue infection till 6

months due to presence of broadly reactive dengue neutralizing antibodies in their mother's serum and the protection being passively transferred passively by maternal antibodies. Here Dengue haemorrhagic fever is explained by enhancing antibodies which leads to an increase in cell mass which is infected. T cell and cytokine response are proportional to this antigenic stimulus. Evidence suggests that antibody dependant enhancement results from idiosyncratic Fc receptor signalling . Resulting production of IL-2 , interferon's and other lymphokines is reinforced by increased abundance of infected target cells resulting from interferon mediated upregulation of Fc receptors and Flaviviral induced expression of MHC type 1 and 2 molecules that further activate T lymphocytes . Activated infected monocytes produce and release TNF alpha , IL-1, Platelet Activating Factor , IL-8 , and RANTES which synergistically with lymphokines , histamines , and viral immune complex induces C3a and C5a to produce the temporary endothelial dysfunction that leads to plasma leakage . After binding to cells, DENV- NS1 antigen induces apoptosis of cells which is mediated by NO. NS1 can activate complement by alternate pathway. The chief abnormality observed in and around small blood vessels consisting of endothelial swelling, perivascular edema and infiltration with mononuclear cells. Cytotoxic factor is a unique pathogenesis associated cytokine in mice and humans seen during dengue virus infections. [39]

Aims & Objectives

AIMS AND OBJECTIVES

1) Importance of NS1 antigen in early detection of dengue fever.

2) Role of IgM/IgG antibody detection in dengue diagnosis.

3) Importance of looking for all three parameters, NS1 antigen, IgM/IgG antibodies in dengue diagnosis.

4) Level of NS1 antigen and severity of dengue fever.

5) Relationship of thrombocytopenia and dengue markers.

6) Comparing Immunochromatographic test and ELISA in the detection of NS1 antigen, IgM/IgG antibodies.

Review of Literature

REVIEW OF LITERATURE

- In 1978, Igarashi A performed virus isolation by inoculation into C6/36 Aedes albopticus cell line[41].

- In 1980, David prepared monoclonal antibodies specific for dengue virus type 3. Mouse lymphocyte hybridomas was prepared by polyethylene glycol – mediated fusion of cells from a mouse plasmocytoma cell line with lymphocytes from a mouse which was hyperimmunised with dengue virus type 3. Media from 50 hybrid colonies was screened; out of which 46 of them showed antibody activity against dengue -3 infected cells as determined by an indirect immunofluorescent antibody technique. Dengue monoclonal antibody which was obtained after cloning one of these colonies also demonstrated activities in haemagglutination inhibition and indirect immuofluorescent antibody assays with dengue 3 antigen, but not with type 1, 2 or 4 antigens. In addition, this antibody activity could be removed from the culture media only by absorption with dengue -3 antigen[42].

- In 1981, Churdboonchart et al for the first time quantified dengue precipitating antibody by a method of inhibition counter current immunoelectrophoresis. This test was also employed to detect dengue virus antibody in patient sera. Anti-dengue type 2 titres determined by inhibition countercurrent immunoelectrophoresis were correlated well with haemagglutination inhibition titres. In secondary cases, more than fourfold increases in precipitating antibodies were observed. The control sera were negative except for sera from a few patients with systemic lupus erythematosus, which had shown low titres.

Simultaneous detection of dengue virus antigen and antibody in the sera collected during acute phase can confirm at least 90% cases. This method was recommended as a routine technique to quantitate antibody in sera from suspected cases of dengue haemorrhagic fever[43].

- In 1983, Burke had reported the serotype specificity of IgM antibody to dengue virus by IgM capture immunoassay with a convalescent –phase serum of four serotypes of dengue virus antigen. He found that serotype specific IgM antibody responses corresponding to the dengue virus type isolated from all the sixteen primary infection patients but only in nine out of sixteen secondary infection patients[44].

- In 1984, Gubler D J and Kuno G identified virus isolates by indirect fluorescent microscopy (IFAT) using serotype- specific monoclonal antibodies[45].

- In 1986 Rice had observed that dengue viral RNA gene is 5' capped and it does not have a poly (A) tail and is translated from a single open reading frame to yield a polyprotien which consists of three structural proteins (core protein C, membrane protein M , and Envelope protein E) followed by seven non structural proteins (NS1 , NS2A, NS2B, NS3 , NS4A , NS4B , NS5)[46].

- In 1989, Winkler etal observed that Dengue NS1 antigen which is a highly conserved 46 kDa non structural glycoprotein exists as an intracellular membrane – associated form and as an extracellular secreted form in the dengue virus infected mammalian cells[47] .

- In 1991 Kuno etal had observed that dengue serology faced with some problems of a small percentage of secondary dengue infected samples with low or no

detectable dengue IgM. Thus a surveillance system that relied on IgM detection alone is not likely to reflect the transmission dynamics of the disease[48].

- In 1992, Blok J etal said that NS1 protein is a 50 kDa glycoprotein which also had similarity among other Flavivirus[49].

- In 1993, Thien et al said that the sera from patients in the acute phase of dengue haemorrhagic fever or dengue shock syndrome (DSS) contained higher levels of anti-dengue antibodies of the IgG1, complement fixing, and subclass than similar sera from dengue fever (DF) patients. Conversely, acute phase sera from DHF and DSS patients also contained lower levels of anti-dengue antibodies of the poor complement activating IgG2 subclass than acute phase sera from DF patients. No significant differences were detected between the levels of anti-dengue IgG3 and IgG4 antibody in acute phase sera from DF, DHF, and DSS patients .With the exception of levels of levels of anti-dengue IgG2 antibody from DHF patients which were lower than those from DF and DSS patients, levels of anti dengue IgG1, IgG2, IgG3 and IgG4 were similar in convalescent sera from all the patients. These results had provided a possible explanation for the activation of the serum complement system which precedes onset of shock in severe dengue infections[50].

- In 1995, Sonali etal did a study on antibody – enhanced binding of Dengue -2 virus to human platelets. The mechanism underlying severe thrombocytopenia in dengue haemorrhagic fever /dengue shock syndrome was not completely understood. Dengue 2 virus binded to human platelets only in presence of virus-specific antibody, supporting a role for immune –mediated clearance of platelets

in the pathogenesis of thrombocytopenia in DHF/DSS. Antibody enhanced binding of virus to the platelets was also demonstrated with a panel of eight murine monoclonal antibodies which was specific for dengue protein [51].

- In 1996, Monath and Heonz had observed that Dengue NS1 antigen can be detected in the circulation during dengue virus infection and it elicits a specific immune response[52].

- In 1996, Mackenzie etal suggested that the function of NS1 antigen in dengue viral replication is not clear. He concluded that NS1 antigen can also serve to anchor the replication complex to the endoplasmic reticulum membrane[53].

- In 1998 Pie-Yun Shu etal had developed envelope and membrane [E/M] non structural protein NS1 serotype –specific capture IgM ELISAs to differentiate four dengue virus serotypes . A total of 93 anti- dengue virus IgM –positive serum samples were collected between day 5 and 45 of illness from 59 confirmed dengue patients were analysed . The results showed that positive serotype specificity could be identified for 86.1 % and 47.6% of serum samples tested for E/M – specific IgM antibodies versus 83.3 % and 42.9% of serum samples tested for NS1 – specific IgM antibodies from patients with primary and secondary dengue infection ,respectively . Dual analysis with both E/M and NS1 serotype – specific capture IgM ELISAs had shown that positive serotype specificity could be correctly identified for 98.6 % and 61.9%of all the primary and secondary serum samples tested , respectively . These findings suggested that E/M and NS1 serotype – specific capture IgM ELISAs have the potential to be of use in dengue virus serotyping[54].

- In1998, Andrea et al had detected dengue antibodies in saliva during dengue infection . Saliva sample was collected prospectively from patients presenting with suspected dengue infection 4 to 8 days after the onset of fever and assayed using a commercial dengue IgM and IgG ELISA. Laboratory diagnosis was based on virus isolation and on haemagglutination inhibition assay. With a positive result defined as either salivary IgM or IgG levels above the cut-off value, an overall sensitivity of 92 % was obtained for both primary and secondary dengue patients, while no patients with non-flavivirus infection and no healthy laboratory donors showed the elevation of salivary anti-dengue antibody. Salivary IgG levels correlated well with the serum Haemagglutination titre and the salivary IgG levels which can be used to distinguish between primary and secondary dengue virus infection[55].

- In 1999, Araujo ESM etal observed a more than fourfold rise in IgG antibody titre against dengue virus by ELISA between acute and convalescent sera samples[56].

- In 2000, Michel Strobel had described 2 patients with autoimmune thrombocytopenic disease who also developed classic dengue fever associated with serious bleeding and extremely low platelet counts (1000 cells/mm^3 3000 cells /mm^3 respectively). These cases had illustrated that the large, harmful and misleading decrease in the platelet count that may be a result of common dengue fever occurring in individuals who co-incidentally have underlying Thrombocytopenic purpura. This situation was not unique, because there are other examples of viral infection which can seriously destabilize chronic cytopenic diseased conditions such as Parvovirus B19 infection in humans, that

cause severe erythroblastopenic anaemia in patients having haemolytic anaemia . Because both patients were adults who used to live in dengue endemic area, they presumably had secondary dengue fever. First, classic dengue fever can induce marked thrombocytopenia in up to 50% healthy subjects with a platelet count that is sometimes as low as 10,000 platelets /mm^3; this feature could not lead to misclassification. Because of air travel becoming more popular these days, emerging dengue fever could not be overlooked. Although Dengue fever may be benign in a large number of healthy subjects, presence of the disease may be of concern in subjects with chronic TCP a relatively common condition[18].

- In 2000, Koraka etal had compared the diagnostic value of immune- complex NS1 antigen dot blot immunoassay (DBI) to a commercially available DEN antigen detection kit and confirmed it with a reverse transcription PCR kit (RT-PCR). 181 serum samples which were obtained from 55 acute dengue virus infected patients was used . From 32 out of 55 dengue patients, viral RNA could be detected by RT PCR. DEN antigen was detected to in only 10 out of 55 patient samples. When these samples were treated with acid release the immune complex associated NS1 antigen for detection by DBI, 43 out of 55 patients were found to be positive dengue NS1 antigen . In non-dissociated samples 22 of these patients were found to be positive by DBI[57].

- In 2000, Young etal had observed that an antigen capture ELISA reveals high levels of dengue virus protein NS1 in the sera of infected patients. This study describes the development of a capture enzyme linked immunosorbent assay for the detection of the dengue virus non-structural, NS1 protein. The assay

employs rabbit monoclonal and polyclonal antibodies as the detection and capture antibodies respectively. Immuno affinity –purified NS1 derived from dengue 2 virus–infected cells was used as a standard to establish a detection sensitivity of approximately 4 ng/ml for an assay employing monoclonal antibodies recognising a dengue2 serotype–specific epitope. A number of serotype cross-reactive monoclonal antibodies were also shown to be suitable probes for the detection of NS1 expressed by the remaining three dengue virus serotypes. Examination of clinical samples demonstrated that the assay was able to detect NS1 with minimal interference from the serum components at the test dilutions routinely used , suggesting that it could form the basis of a useful additional diagnostic test for dengue virus infection . The quantitation of NS1 levels in the patient sera may prove to be a valuable surrogate marker for viraemia[58].

- In 2001 , Kurane. I demonstrated that the anti-NS1 antibodies are mainly formed during secondary dengue viral infections in patients from Indonesia (where dengue haemorrhagic fever is more common than in patients from Caribbean where dengue haemorrhagic fever is less common). These pre-existing anti-NS1 antibodies possibly result from the formation of antigen –antibody immune complexes and therefore reduce the sensitivity of DEN NS1 antigen detection assay[22].

- In 2002, Libraty etal observed that an increased levels of NS1 antigen were formed within 72 hours of illness, which identified the patients at risk of developing dengue haemorrhagic fever , through the quantitative estimation of NS1 antigen were not carried out in this study to confirm the diagnosis[59].

- In 2002 Chow etal had suggested that NS1 antigen detected in 82% to 83% of dengue patients from the first day of fever up to the ninth day from the onset of fever[60].

- In 2002 et al Wang et al detected replication of dengue virus in the peripheral blood mononuclear cells from dengue virus type 2 –infected patients by a reverse transcription– real– time PCR assay. While dengue virus is thought to replicate in mononuclear phagocytic cells in vivo, attempts had been done to detect it in peripheral blood mononuclear cells (PBMC) by virus isolation or antigen detection have had variable and generally low rates. In this study, a reverse transcription (RT)- real time PCR assay was developed to quantify positive – and negative –sense RNA of dengue virus type 2 within the cells . The assay includes an RT step using either sense or antisense primer followed by a real –time PCR step using the designed primers and probe , which could target a capsid region which is highly conserved in dengue virus type 2 strains . It can be used to monitor the dynamic change of intracellular dengue virus RNA species during the course of infection. When this assay is employed in quantification of dengue virus RNA species in PBMC from 10 patients infected with dengue virus type 2, both positive and negative sense dengue RNA can be detected, indicating that dengue virus is actively replicating in PBMC in vivo. Moreover, the amounts of negative–sense dengue virus RNA in PBMC correlate very well with the viral load of dengue virus replication in vivo[61].

- In 2003, Koraka P etal , NS1 detection rate is higher in patients with primary dengue than those with secondary dengue viral infections . A possible basis for reduced sensitivity in secondary infection is that NS1 along with other viral

antigens is sequestrated in immune complex when a substantial level of the dengue viral reactive IgG is present[57].

- In 2003, Gubler had said that Dengue fever which is mosquito borne viral disease affects approximately 50-100 million individuals yearly in more than hundred countries [62].

- In 2002, Diana used recombinant antigens for differentiation of dengue virus serotype 1 to 4 by serology . The B domains of dengue virus serotypes 1 to 4 were expressed in Escherichia coli. The purified proteins applied to immunoblot strips to detect serotype specific antibodies in paired serum samples from 41 patients with primary and secondary dengue viral infections. A close correlation was found between the results obtained with the immunoblot strips and by type specific RT- PCR[22] .

- In 2002, Alcon etal had demonstrated that IgM ELISA, which is commonly used assay, has decreased sensitivity in the first 4 day of illness[63].

- In 2002, Twiddy investigated genetic diversity and phylogenetic relationships of a collection of strains of dengue virus type 1 (DV-1) isolated from different parts of the world. Phylogenetic trees derived from the complete sequence of the E gene of suggested the existence of five genetic types defined by the maximum nucleotide divergence within each group of 6%. The average rate of evolution was estimated to be approximately 16.2 $x10^{-4}$ substitutions third codon position site year. Using this estimate, divergence among the DV-1 genotypes was calculated to have occurred approximately 100 years ago. Very low average value of the ratio of non-synonymous –to-synonymous nucleotide substitutions ,

relative to the respective sites (0.046), indicated that the evolution of the E gene of the DV1is subject mostly to purifying selection . Nucleic acid sequence comparison revealed that the identify among the DEN-4 viruses was greater than 92%. Similarly among deduced amino acids was between 96 and 100%; in most cases identical amino acid substitutions occurred among viruses from similar geographical regions. Recent dengue evolution in Puerto Rico can be attributed in part to positive selection on the non structural gene 2A (NS2A) whose function may include replication efficiency and antigenecity. During the latest and most severe DEN-4 epidemic in 1998, viruses were distinguished by three amino acid changes in NS2A that were fixed far faster than expected by drift alone . It therefore demonstrates viral genetic turnover within a focal population and the potential importance of adaptive evolution in viral epidemic expansion .A number of amino acid positions have been identified with the envelope (E) glycoprotein that is subjected to relatively weak positive selection in both DEN-3 and DEN-4 , as well as in two genotypes of DEN-2 . No positive selection was detected in DEN-1 . In accordance with the function of the E protein as the major antigenic determinant of DEN, the majority of these sites were located in, or near to, potential T- B- cell epitopes[64].

- In 2002, Woelk etal Several positively selected amino acid substitutions were also identified in the NS2B and NS5 genes of DEN-2 , although the cause of this selection is unclear, whereas the capsid, membrane and non-structural genes NS1 , NS2A , NS3 and NS4 were all subjected to strong functional constraints . Hence, evidence was found for localized adaptive evolution in

natural isolates of DEN, revealing that selection pressures differ among serotypes , genotypes and viral proteins[65] .

- In 2002, Wang etal detected replication of dengue virus in peripheral blood mononuclear cells from Dengue virus type 2 infected patients by a reverse transcription – Real –Time PCR Assay. While dengue virus is thought to replicate in mononuclear phagocytic cells in vivo, attempts to detect it in peripheral blood mononuclear cells (PBMC) by virus isolation or antigen detection have had variable and generally low rates. In this study, they developed a reverse transcription (RT) - real time PCR assay to quantify positive and negative sense of dengue virus type2 within the cells. The assay include an RT step using either sense or antisense primer followed by real time PCR using the designed of intracellular dengue virus RNA species during the course of illness[61].

- In 2002 and2003 Hua Xu etal had observed that the rapid diagnosis and serotyping of dengue viral infections was important for timely diagnosis and management in areas where multiple flaviviruses was epidemic. A specific antigen captures ELISA for early detection and serotyping of dengue virus serotype1 by using well characterised monoclonal antibodies (MAbs) specific to monoclonal antibodies specific to NS1 of dengue virus 1 was developed . With this assay 462 serum specimens from clinically probable dengue virus 1 infected patients during dengue virus 1 epidemics in Guangdong, China in 2002 and 2003 were analysed. Dengue virus NS1 was detectable in the blood circulation from the first day up to 18 days after the onset of symptoms with a peak at days 6 to 10. The sensitivity of DV1 NS1 detection in serum specimens

with reference to results from reverse transcriptase PCR was 82 % and the specificity was 98.9% with reference to 469 healthy donors[66].

- In 2004, Schilling S said that immune response in primary dengue infection have been characterised by a slow and low titre of IgG antibody response. IgM antibodies appear only 3 to 5 days after the onset of the disease. Thus there is a transient window period of first few days of illness if antibody is used as a diagnostic test[18].

- In 2004, Wagennar said that Human leucocyte antigens (HLA) expressed on the cell surface function as antigen presenting molecules and those polymorphism could change individual's immune response . A study conducted in Viatnamese children had shown that persons carrying HLA-A*240203/10 are about 2 times more likely to have severe dengue infection than others. On the other hand, HLA-DRB*10901 persons are less likely to develop DSS with DEN-2 virus infection. These results clearly stated that HLA controlled the susceptibility to severe forms of dengue viral infections[67].

- In 2004 Pie-Yun Shu said that virus isolation from mosquitoes remained the gold standard although it has been replaced by RT-PCR for rapid. The dengue virus can be successfully detected by culture but viraemia is brief. Within a day or two after subsidence of fever, antibodies rise and further interfere with virus isolation. Dengue virus is thermolabile. Viral laboratories are expensive to develop and maintain and services are not widely available .The common sample used for culture include serum, plasma, leukocytes washed of antibodies from patients; homogenised minced tissues eg, liver, lung , spleen, lymph nodes,

thymus, cerebrospinal fluid , serum and plasma as autopsy specimen. Early collection of sample, proper handling and prompt delivery to the laboratory leads to successful isolation of virus. Isolation may be done by inoculation into the thorax of mosquitoes belonging to Toxorhynchites spendens or adult male mosquitoes of Aedes aegypti or Aedes albopticus . Although they were smaller in size, require more delicate inoculation technique, they have sensitivity equal to that of Toxorhynchites. Specimen may also be inoculated in any of the widely available mosquito cell lines (eg; C6/36, AP-61, Tra-284, AP-64 CLA-1 cell lines , and mammalian cell like LLCMK2, Vero and BHK21). This technique was slightly sensitive than inoculation in live mosquitoes. Culture in cell lines like VERO and in intracerebrally inoculated newborn mice is the least sensitive methods. Dengue type specific monoclonal antibodies are used to examine mosquito head squashes, infected cells, and infected cell culture fluids for identification. The binding of specific monoclonal antibody is revealed by a second labelled antibody (indirect fluorescent antibody test using serotype antibodies)[68].Progress toward the detection of antigen in acute –phase serum samples by serology has been slow due to the low sensitivity of the assay for the patients with secondary infections, as such patients have pre-existing virus-antibody immunocomplexes. However , recent studies used ELISA and dot blot assays directed to the EM antigen (the Denkey kit ; Globio Co. , Beverly , Mass) and the NS1 antigen demonstrated that high concenterations of the EM and NS1antigen demonstrated that high concenterations of the E/M and NS1 antigens in the form of an immune complex could be detected in the acute – phase sera of both patients with primary dengue virus infections and patients

with secondary dengue virus infections up to 9 days after the onset of illness. Koraka et al recently reported on the detection by a dot blot immunoassay of immune complex –dissociated NS1 antigen in patients with acute dengue virus infections. Although it is demonstrated the potential of NS1 antigen detection for the serodiagnosis of acute dengue virus infection , the relatively low rate of positive results by the RT-PCR assay reported in the study suggested that it was under estimated . In addition, many RT-PCR-positive samples showed negative results when they were analyzed by this NS1 antigen detection method. The serological diagnosis of dengue virus infection is rather complicated for the following reasons : 1) patients may have multiple and sequential infections with four dengue virus serotype due to lack of cross-protective neutralisation antibodies, 2)multiple and sequential flavivirus infection make differential diagnosis difficult due to the presence of pre-existing antibodies and original antigenic regions were two or more flavivirus are co circulating 3) IgG antibodies have high degrees of cross- reactivity to homologous and heterologous flavivirus antigens ; and 4) the serodiagnosis of past , recent , or present dengue infections is difficult due to prolonged persistence of IgG immunoglobulin's (>/= 10 months , as measured by E/M specific capture IgG ELISA , or lifelong , as measured by E/M antigen-coated indirect IgG ELISA) in many dengue patients with secondary infections . Thus among the dengue viral infections that can be diagnosed by serological methods is the most challenging. Several methods have been described for the serological detection of dengue virus – specific antibodies , including the haemagglutination inhibition (HI) test , the neutralisation test , the indirect immunofluorescent –

antibody test , ELISA, complement fixation , dot blotting , western blotting and the rapid immunochromatography test (for which many commercial kits are available). Among these, capture IgM and /or IgG ELISA , antigen coated directed IgM and /or IgG ELISA and the HI test are commonly used serological techniques for the routine diagnosis of dengue viral infections. Traditionally the HI test was used to differentiate between primary and secondary dengue infections due to its simplicity, sensitivity, and it was reproducible. Patients were classified as secondary dengue virus infections when the HI test titre in their sera is greater than or equal to 1:2,560 and were classified as having primary dengue virus infection if the HI test titre is less than 1:2,560. The HI test is now less popular and has gradually been replaced by the E/M-specific capture IgM and IgG ELISA. The E/M –specific capture IgM and IgG ELISA has become the most powerful assay for the serodiagnosis of dengue virus infection due to its high sensitivity, specificity , simplicity , and feasibility . Anti-dengue virus IgM immunoglobulins may be produced transiently during primary and secondary infections. In patients with primary dengue infections, IgM immunoglobulin's develop fast and are detectable on days three to five of illness in half of the hospitalised patients .Studies have shown that anti-dengue virus IgM levels peak at about 2 weeks post infection and then decline to undetectable levels over 2 to 3 months . Anti-dengue virus IgG appears shortly afterwards[28].

- In 2004, Sergio et al published a journal on the laboratory tests that the clinician must know to reach a correct diagnosis. In Brazil, the increase in the incidence of dengue was especially linked to the dissemination of Aedes aegyptii . Thus a

rapid and accurate dengue diagnosis was of paramount importance for effective control of dengue outbreaks. Five serological test have been used for the diagnosis of dengue infection; haemagglutination inhibition (HI), complement fixation (CF), Neutralisation test (NT) , IgM ELISA and indirect IgG ELISA. The limitation of the above test are high degree of cross reactivity observed with these tests. Four methods of viral isolation had been routinely used for dengue viruses; intracerebral inoculation of newborn mice, inoculation on mammalian cell cell cultures, intrathoracic inoculation of adult mosquitoes, and inoculation on mosquito cell cultures . In recent years, several new diagnostic techniques have been developed and have proved very useful in dengue fever diagnosis , such as; nucleic acid hybridisation , RT-PCR . Currently, dengue diagnosis is based on serology, viral isolation and RNA detection[69].

- In 2004, Innis etal classified primary and secondary dengue infections by determining the ratio of the units of dengue virus IgM antibodies to the units of dengue virus antibodies to the dengue virus IgG antibodies. They had shown that acute-phase sera of patients with primary dengue virus infections had higher IgM/IgG ratios, where as patients with secondary infections had lower IgM/IgG ratios .This method had made a great contribution to the analysis of the immune status of the patient with dengue. Studies have shown that most patients with primary dengue virus infection would not have detectable IgM or IgG antibodies at 4 months post-infection. This is in contrast to the high IgG titres which was detected in many of the patients with secondary dengue even 10 months after infection[70].

- In 2004, Ahluwalia studied about the earlier principal vector of dengue virus in Asia, Aedes albopticus which has been replaced by Aedes aegypti. Interestingly, Aedes aegypti had been described as having a relatively low oral receptivity for dengue virus as to Aedes albopticus .However , it has been recently documented that oral receptivity of Aedes aegypti to DEN-2 virus was significantly more than Aedes albopticus . Aedes aegypti tends to be more susceptible to infection by DEN-2 virus of South-east Asian genotype as compared to American genotype. These observations obviously have important epidemiological implicationsepidemiological implications for Asian countries as the local vector has increased propensity to transmit dengue infection , especially DEN-2 (relatively more virulent serotype)[71] .
- In 2005, Kao L and Teles FR proposed that the IgM immunoglobulin response varies considerably among persons due to different host immune response [70].
- In 2005, Ngyugen et al have shown that there was no considerable association between sex, nutritional status of the patient and the severity of Dengue haemorrhagic fever and dengue shock syndrome[72].
- In 2005, Severine et al had used four dengue viral antigens for the determination of dengue immune status by ELISA of IgG avidity. IgG ELISA were used to determine the dengue immune status of 105 pairs of serum samples from patients infected with dengue virus. This study had shown that a simple avidity test, for which only one acute – phase serum sample is required , is potentially more useful than haemagglutination inhibition test for the discrimination of primary from secondary infection , whatever the type of dengue antigen is used[73] .

- In 2006, Lin CF suggested that anti-NS1 immunoglobulin's induce endothelial cells to undergo apoptosis and invitro experiment had demonstrated that these antibodies are responsible for increased permeability in endothelial cell monolayer[74].It is worth emphasizing that although E/M-specific IgG antibodies are highly cross reactive among various flaviviruses during a secondary response , the E/M and NS1 –specific anti-dengue virus IgM antibodies have limited cross-reactivities. Therefore, the crossreactivity of dengue-specific IgM antibodies found in a few dengue patients may actually indicate a recent infection with another flavivirus . Some investigators have studied dengue virus –specific IgA and IgE antibody responses. Talarmin et al reported on the use of an IgA and IgM specific capture ELISA for the diagnosis of dengue virus infection. The results showed that IgM appears more rapidly and lasts longer than IgA .They concluded that the capture IgA ELISA is a simple method that can be performed together with the capture IgM ELISA and that can help in interpreting the serology of dengue fever[56] .

- In 2006 Balmaseda et al reported that the dengue specific IgM and IgA antibodies can be detected in serum and saliva of dengue infections. They concluded that that dengue virus –specific IgA in serum can be a potential diagnostic target[53].

- In 2006, Dussart P etal , suggested that NS1 antigen is chemically a glycoprotein that is essential for the viability of dengue virus and it is produced in two forms ;membrane associated and secretary forms. He also observed that Enzyme linked immunoassays directed against NS1 antigen have demonstrated

its presence in high concenterations in the sera of dengue virus infected patients during the acute phase of the disease[75].

- In 2006 to 2007 , Maria G. Guzman did a multicountry evaluvation of the sensitivity and specificity of two commercially available NS1 ELISA assays for dengue diagnosis. This study suggested that the best performing NS1 assays had a moderate sensitivity (64 %) and a high specificity of 100 % for the dengue diagnosis . The combination of NS1 and IgM detection in samples collected in the first few days of fever increased the overall dengue diagnostic sensitivity[76].

- Nunes etal in 2006 and Chem YK etal in 2007 suggested that detection of secreted NS1 protein represents a new approach to the diagnosis of acute dengue infection . A recently developed commercially available diagnostic test based on dengue NS1 antigen capture ELISA was investigated in two studies (one in South America and the other in South-east Asia). The test had an overall sensitivity of 88.7 % and 93.4 % in the two studies, with 100% specificity[77,78].

- In 2006, Ravulapalli et al studied recombinant multiepitope protein for early detection of dengue infections . A customised recombinant dengue multiepitope protein (r-DME-G) that can specifically detect the immunoglobulin G (IgG) class of anti- dengue antibodies in patient sera was developed. Using this strategy, another dengue multiepitope protein, r-DME –M with specificity for IgM class of anti-dengue antibodies. A synthetic gene encoding the r-DME-M was expressed as a maltose –binding fusion in Escherichia coli. The recombinant protein was purified in a single affinity chromatographic step to obtain yields of 15 mg purified protein/litre of culture. The purified protein was

used to develop an in-house IgM ELISA and tested using a panel of 172 patient sera characterised using the commercially available Dengue Duo rapid strip test from Panbio , Australia . The IgM ELISA results showed that the r-DME-M protein not only recognised all IgM samples identified by the Panbio test but also identified samples missed by the latter test[37].

- In 2006, Herman et al had studied the extent to which immune responses contribute to the long term protective immunity afforded by natural primary DENV infection has not been fully defined . Neutralisation of infection in monocytic cells (instead of enhancement of infection) may be more strongly associated with protection from DHF, although this association has been reported in only one study. Humans can experience three sequential heterologous dengue infections. Importantly, the occurrence of a second and third infection in individuals with pre-illness antibodies against multiple dengue serotypes indicates that neutralizing antibodies are cross- reactive invitro but not cross-protective in vivo[79].

- In 2006, M Moorthy has conducted a study to evaluate the sensitivity of rapid immunochromatographic test (ICT) device in detecting antibodies to dengue virus in tertiary hospital in South India . Sera from hospital attendees, with requests for dengue virus antibody testing were tested with Dengue duo cassette and a reference antibody capture assay for detection of IgM and IgG antibodies. The ICT results were compared with results of antibody capture tests for detection of IgM and IgG antibodies respectively. Accuracy indices for IgM and IgG detection respectively were; sensitivity 81.8% and 87.5%, specificity 75 % and 66.6% , positive predictive value (PPV) 61% and 72.9% and negative

predictive value 89.6% and 83.9% . The device performs poorly in detection of IgM and IgG antibodies to dengue viral infections and is not recommended for use as a standard –alone diagnostic procedure[80].

- In 2007, Uma Reddy et al reported that dengue viral infection specifies the activation of unfolded protein response . Recently, virus induced apoptosis mediated by the unfolded protein response (UPR) has been hypothesised to represent a crucial pathogenic event in viral infection . In an attempt to evaluate the contribution of the UPR to virus replication , They have characterized each component of this signalling pathway following dengue virus infection . This study proved that dengue virus infection, A549 cells elicits an UPR which is observed at the level of translation attenuation and activation of specific of specific pathways such as nuclear translocation of ATP-6 and splicing of XBP-1. Interestingly, the study found that specific serotype of virus modulate the UPR with different selectivity. In addition we demonstrate that perturbation of the UPR by preventing the dephosphorylation of the translation initiation factor elf2alpha using Salubrinal considerably alters virus infectivity. This report provides evidence that dengue infection induces and regulates the three branches of the UPR signalling cascades. This is a basis for our understanding of the viral regulation and conditions beneficial to the virus infection . Furthermore , modulators of UPR such as Salubrinal that inhibit dengue replication can open up an avenue toward cell- protective agents that target the endoplasmic reticulum for anti-viral therapy[70] .

- In 2007, Puttikhunt etal demonstrated that secreted NS1 antigen attaches to the surface of the cells via interactions with heparin sulphate and chondroitin

sulphate. This study also demonstrated that soluble NS1 protein binds to endothelial cells and following recoginition by anti-NS1 antibodies, could contribute to plasma leakage during severe dengue viral infection[81].

- In 2007, Kurosu et al suggested that patients with an elevated level of free soluble form of dengue virus (DV) non structural protein 1 (sNS1) are at risk of developing dengue haemorrhagic fever. Complement inhibitory factor Clusterin (Clu), which naturally inhibits the formation of terminal complement complex (TCC) was identified by mass spectrophotometry . Since an activated complement system reportedly cause vascular leakage, the interaction between NS1 and Clu may contribute to the progression of dengue haemorrhagic fever [82].

- In 2007 Koraka et al reported that the capture IgE ELISA can detect total and dengue virus-specific IgE antibody responses . The results showed that dengue virus specific IgE titres were significantly higher in patients with dengue fever and non-dengue virus infections. They concluded that analysis of dengue virus specific IgE ELISA might contribute to the understanding to the understanding of the pathogenesis of dengue virus [83].

- In 2007, Sierra studied Cuban DHF/DSS outbreaks .This study had provided evidence of a reduced risk of people of Negroid race for DHF/DSS compared to those of Caucasoid race. Taking into consideration the origins of contemporary Cuban inhabitants, the Cuban, Carribean Black and African populations share a common gene pool that could explain, at least partially, the low incidence of dengue haemorrhagic in Cuba and Caribbean and African countries. The central role played by immunological mechanisms in the pathogenesis of DHF/DSS

has led to consider that the polymorphic genes associated with immune response must be carefully considered among those human genes regulating regulating dengue disease severity that might be distributed unequally in Blacks and Whites[84] .

- In 2007, Avirutnam P etal said that NS1 levels can enhance complement activity in solution and or by directly binding endothelial cells and may establish foci for immune complex formation leading to complement activation , endothelial damage and capillary leakage[81].The virus which is found in the serum or plasma , for 2-7 days , corresponding to the days of fever . Person never previously infected with a flavivirus nor immunised with a flavivirus vaccine , mount a primary antibody response when infected with the virus .The dominant immunoglobulin isotype is IgM . The haemagglutination inhibiting antibody appears simultaneously with IgM antibody. IgG appears shortly afterwards. Individuals with immunity immunity due to previous flavivirus infection or immunisation mount a secondary (anamnestic) antibody when infected with dengue virus. Here the dominant immunoglobulin type is IgG. IgM appears in most instances, but its levels are lower than that observed in primary infections[70].

- In 2007, Sekaran had suggested that the NS1 antigen detection rate decreases as level of IgM antibody increases [85].

- In 2007, a special issue on Dengue news letter described about feeding habit about the vector Aedes aegypti which is a day biter with increased biting activity 2 hours after sunrise and several hours before sunset, feeds in domestic

and peridomestc situations . It bites repeatedly. They rests in the domestic and peridomestic situations in the dark corners of the houses , on hanging objects like clothes , umbrella or under the furniture . Aedes aegypti mosquito breeds in any type of man made containers or storage containers having even a small quantity of water .Eggs of Aedes aegypti can live without water for more than one year. Desert coolers, drums, jars, pots, buckets, flower vases, lant saucers, tanks, cisterns, bottles, tins, tyres, roof gutters, refrigerator drip, pans, cement blocks, cementary urns, bamboo stumps, coconut shells, tree holes and many more places where rainwater collects or is stored[32] .

- In 2008, Bess of K suggested that NS1 antigen circulates uniformly in all types of dengue viral infections and it circulates at high levels during the first few days of illness [86].

- In 2008, Arthur reported that the live attenuated vaccines had been developed for dengue which could provide an economically viable approach to inducing durable humoral and cellular immunity. Neutralising antibodies are known to mediate protection from re-infection with a homologous serotype, and long term immunity to dengue viruses has been described. The virus must be able to replicate sufficiently well in vivo to provoke an immune response, but must be sufficiently restricted in systemic replication to avoid inducing any of the dengue-associated symptoms. The vaccines need to induce a broad neutralising antibody response against each serotype, thus avoiding preferential antibody reponses to the better replicating components, leading to disease susceptibility. For over twenty years, monovalent live attenuated vaccine candidates leading to disease susceptibility. For over twenty years monovalent live attenuated vaccine

candidates, propagated and attenuated in primary and diploid cell cultures had been evaluvated in human beings[87].

- Halstead and Marchette made the first successful monovalent vaccine using primary dog kidney cells. Several monovalent vaccines passaged through primary dog kidney cells were developed and combined into several tetravalent formulations that have been tested in phase 1 and 2 trials in American adult volunteers or Thai adults and children where they were shown to be safe and immunogenic[22] .

- In 2008 , Lertwongrath S etal proved that if NS1 antigen detection in acute phase sera is combined with dengue viral IgM detection in early convalescent phase sera , a sensitivity of about 90% in dengue diagnosis could be obtained[88].

- In 2008, Shrivastava etal established that NS1 antigen ELISA can be used in early diagnosis of dengue viral infections. Out of 91 clinical samples 26 % were positive by NS1 antigen capture ELISA, 16% positive by SD Dengue NS1 antigen test and 12% positive by RT-PCR analysis[15].

- In 2008, Kumarasamy V etal said that dengue early ELISA has the added advantage of giving a good detection rates up to 7 days of illness[89].

- 2008, Jose reported the ophthalmic complications in dengue infections are rare. The main ocular findings include subconjunctival haemorrhages, macular oedema and blot haemorrhages. Less common features include exudative retinal detachment, cotton wool spots, glaucoma and anterior uvietis. A majority of patients were reported to have residual visual impairment[90].

- In 2008, Chien had described about the iatrogenic complications include water intoxication, sepsis due to contaminated iv lines, pneumonia, wound infections, heart or respiratory failure due to over hydration. Simultaneous infections such as leptospirosis, viral hepatitis, typhoid fever, chicken pox, meliodosis had been reported and could contribute to unusual manifestation[91].

- In 2008, Webster reported that subunit vaccine have poor immunogenicity and need potent immunostimulatory adjuvants that activate the innate immune system and there by elicit antigen-specific immune responses. Monophosphoryl lipid A is a detoxified form of the endotoxin lipopolysaccharide, and is among the first of a new generation of toll –like receptor agonists likely to be used as vaccine adjuvants on a mass scale in human populations. A recombinant subunit protein containing the amino-(N)terminal 80% of the DENV2 envelope protein had been studied in formulations with one of the five different adjuvants based on aluminium, fractions QS-21 of saponin , and monophosphoryl lipid A. In mice, all formulations were immunogenic with evidence of non-sterilising protective efficacy. A new tetravalent protein vaccine candidate has been developed that comprises a consensus dengue virus envelope dengue virus protein domain III (cEDIII) obtained by alignment of aminoacid sequences from different isolates of all four serotypes. Formulation of recombinant Ced111 with aluminium phosphate induces cross-reacting antibodies and memory immunity after challenge with dengue virus in mice. DNA vaccines use plasmids of double-stranded DNA in which the genetic sequence for a protein or series of epitopes has been inserted under the control of a mammalian promoter. They are usually given intramuscularly and are taken up by muscle and dendritic cells,

where the genetic information for the encoded protein is translated . Several dengue DNA vaccines have been developed. Although DNA vaccines showed early promise in other disease models in animals, DNA vaccination generally elicits only weak immune responses in people although they are effective at priming cellular immune responses, including those of T-helper -1 type CD4 T cells and CD8 cytotoxic T cells, for boosting by recombinant viruses[70].

- In 2008, S Datta has undertaken a study to evaluvate the efficacy of NS1 antigen assay as an early marker for dengue virus infection Group I evaluvated the performance of NS1 antigen assay in comparison to MAC- ELISA and their detection rate when performed together in a single sample. Six hundred acute / early convalescent sera were screened by both assays. Group II evaluvated the efficacy of a single assay in 30 acute phase sera of pediatric OPD patients screened only by NS1 antigen assays. Group III evaluvated the specificity of NS1 assay in comparison to MAC- ELISA on 40 samples included as controls. In Group 1 23% and 39% were positive by NS1 antigen assay and MAC – ELISA respectively .The detection rate increased to 53 % when both the assays were used together on a single sample. NS1 antigen positivity varied from 71 % to 28 % in acute and early convalescent sera, conversely IgM detection rate was 93% and 6% in early convalescent and acute phase sera respectively. In Group 11 66 % samples were positive by NS1 assays. The above study shows that NS1 antigen assays holds promise in early dengue infection [8].

- In 2008 Dussart etal did a study which compared the performance of two new commercial test kits for detection of NS1 antigen during the clinical phase of dengue virus infection - an immunochromatographic test allowing rapid

detection of NS1 antigen , Dengue NS1 antigen strip , and a two step sandwich format microplate ELISA , the Platelia Dengue NS1 Ag test.272 serum samples from patients with dengue disease were tested . Of these 222 were from patients with acute infection with one of the four serotypes of dengue virus, detected by RT PCR and or virus isolation .Fourty-eight acute phase serum samples from patients not infected dengue virus were also included.The sensitivity of Platelia Dengue NS1 Ag test on acute serum samples was 87.4 %; that of Dengue NS1 Ag strip was 81.5 % after 30 minutes. Both tests had specificity 100%. The Pan –E Dengue Early ELISA had a sensitivity of 60.4 % and a specificity of 97.9%. These findings support the use of NS1 antigen detection for the diagnosis of acute dengue viral infection. The immunochromatographic was highly sensitive and specific, and would therefore be a suitable first line test in the field.

- In 2008, Datta evaluvated the NS1 antigen assay for early diagnosis of dengue viral infection . Group I evaluvated the performance of NS1 antigen assay in comparison to MAC-ELISA and their detection rate when performed on a single sample .Six hundred acute/early convalescent sera were screened by both assays. Group11 evaluvated the efficacy of a single assay in 30 acute phase sera of pediatric OPD patients screened only by NS1 antigen assay. Group III evaluated the specificity of NS1 assay in comparison to MAC-ELISA on 40 samples included as controls. In Group 1, 140 (23%) and 235 (39%) samples were positive by NS1 assay and MAC-ELISA respectively. The detection rate increased to 53 % when both assays were used together. From this study it was concluded that NS1 antigen assays holds promise in early diagnosis of dengue viral infections[9].

- In 2008, Vu Hang etal suggested that NS1 assays can be included in the diagnostic evaluvation of dengue patients, but with due consideration for the limitations in patients, who present late in their illness or have a concomitant humoral immune response. The sensitivity and specificity of NS1 ELISA and a NS1 lateral flow rapid test were compared against a gold standard reference diagnostic algorithm 138 Vietnamese children and adults. Overall, NS1 ELISA was modestly more sensitive (82%) than NS1 LFRT (72%) in confirmed dengue cases[92].

- In 2009, Subhash C Arya reported NS1 detection in addition to reverse transcriptase PCR and Transcription mediated amplification of dengue virus in acutely ill patients .The study have attempted to determine the utility of RT-PCR in relation to the appearance of the DENV NS1 antigen . NS1 antigen detection appeared to be better than RT-PCR for eg, ports in Taiwan was instrumental in the detection of 19RT-PCR-negative travellers who would have been labelled DENV negative . Two of them turned out to be IgM positive on the day 17 or day 18 of illness[93].

- In 2009 , Vazquez etal reported the application of monoclonal antibody to dengue capsid protein in dengue studies[22] .

- In 2010, Aryati et al did a study on the performance of dengue NS1 antigen ELISA and molecular analysis of NS1 gene of dengue viruses obtained during surveillance in Indonesia. The NS1 and IgM/IgG ELISA assays were used for screening and confirmation of dengue infection during the surveillance in 2010-2012. Collected serum samples (n=440) were subjected to RT-PCR with virus isolation in which 188 samples were confirmed for dengue infection. The

positivity of the ELISA assays were correlated with the RT-PCR results to obtain the sensitivity of the assays. The NS1 genes of 48 Indonesian birus isolates were sequenced and their genetic characterstics were studied. Using molecular data as the gold standard , the sensitivity of NS1 ELISA assay for samples from Indonesia was 56.4 % while IgM ELISA was 73.7% .When both NS1 and IgM results were combined , the sensitivity increased to 89.4 % . The NS1 sensitivity varied when correlated with city /geographical origins and DENV serotype, in which the lowest sensitivity was observed for DENV-4 . NS1 sensitivity was higher in primary compared to secondary infection . The specificity of NS1 assays for the non-dengue samples were 100%. The NS1 gene sequence analysis of 48 isolates revealed the presence of polymorphism of the NS1 genes which did not influence the NS1 sensitivity[94].

- In 2010, Subhash C Arya, conducted a study when there was increase in dengue viral infections in New Delhi. This study was conducted at Sant Parmanand Hospital during this outbreak to determine the utility of Dengue package, comprising simultaneous detection of dengue NS1 protein , anti-dengue IgM antibodies, anti-dengue IgG antibodies and platelet enumeration. Blood samples were tested for dengue NS1 antigen, IgM and IgG antibodes using single step immunochromatographic test. Of the 1886 patients screened with dengue package, 678 were NS1 positive . The platelet count was lower in NS1 positive than in NS1 negative cases [93].

- In 2010, Thomas etal has suggested that the dengue viral NS1 antigen (that develops right at the beginning of feverish period and before appearance of dengue antibodies), is emerging as a suitable option for dengue diagnosis[22].

- In 2010 Seok etal had evaluvated a commercial dengue virus NS1 Antigen Capture Enzyme Linked Immunosorbent Assay kit for early diagnosis of dengue viral infection. A total of 399 serum samples in comparison with real time PCR , an in-house IgM capture ELISA (MAC-ELISA) and an haemagglutination inhibition assay. Of the 320 dengue sera , 64% tested positive for NS1 antigen compared to 93% by either MAC-ELISA or RT-PCR , 50% by RT-PCR and 70% by MAC-ELISA only. The NS1 detection rate was inversely proportional while the IgM detection rate is directly proportional to the presence of IgG antibodies. The overall sensitivity and specificity of NS1 antigen ELISA in the detection of confirmed dengue virus sera are 76.76 % and 98.31 % respectively. This suggests that NS1 antigen ELISA has a high degree of sensitivity and specificity for detection of dengue viral infection[95].
- In 2010, Guzman et al has hypothesised that the sensitivity of NS1antigen test can differ according to dengue virus serotype and a reduced sensitivity of NS1 assays for DENV-2 serotype[37] .
- In 2010, Alvina etal found a lower sensitivity of protein tests during a dengue type 2 virus outbreak in Santos , Brazil. The detection of NS1 antigen was used for diagnosis in addition to the detection of IgG , IgM and RNA , A large number of NS1 false negative results were obtained , A total of 379 RNA positive samples were selected for thorough evaluvation . NS1 was reactive in 37 % of cases .Most of the cases were characterised as a secondary dengue infection by dengue virus 2 [18].
- In 2010, Alvina et al observed low sensitivity of NS1 protien tests evidenced during a dengue type 2 virus outbreak in Santos, Brazil, in 2010.

- In 2010, Shamala etal used commercial Dengue Duo rapid test kit for early diagnosis by detection of dengue virus NS1 antigen and immunoglobulin IgM /IgG antibodies . A total of 420 serum samples were subjected to real time PCR, in-house IgM capture ELISA, haemagglutination inhibition assay and the SD Dengue Duo rapid test. Of the 320 dengue acute and convalescent sera, dengue infection was detected by either serology or by RT-PCR in 300 samples as compared with 289 samples in the combined SD Duo NS1 /IgM . The NS1 detection rate is inversely proportional whereas IgM detection rate is directly proportional to the presence IgG antibodies. By this study it was shown that detection of both NS1 and IgM by SD duo gave comparable detection rate by either serology or by RT-PCR[95].

- In 2010 , Vianney et al compared the sensitivity, specificity and relationship to viraemia of two dengue NS1 rapid tests. The NS1 rapid tests had similar diagnostic sensitivities in confirmed dengue cases but were 100 % specific . When IgM/IgG results from the SD Dengue Duo were included in the test interpretation , the sensitivity improved significantly from 62.4 % with NS1 alone to 75.5 % when NS1 and/or IgM was positive and 83.7% when NS1 and /or IgM and /or IgG was positive . Both NS1 assays were significantly more sensitive for primary than secondary dengue. NS1 positivity was associated with the underlying viraemia as NS1 –positive samples had a significantly higher viraemia than NS1 – negative samples[96].

- In 2011, Pallavi etal reported on the rational use of platelet transfusion in dengue fever. The practise of platelet transfusion has been adapted into standard clinical practise in management of hospitalised dengue patients. The exact

indications and situations in which platelet have to be transfused may vary greatly. Blood components especially platelet concenterates due to their short shelf life are frequently in limited supply. Hence appropriate use of blood is required to ensure the availability of blood for patients in whom it is really indicated, as well as to avoid unnecessary exposure of the patients to the risk of transfusion reaction and blood borne infection. Clinical data from 343 serologically confirmed dengue patients admitted at JSS University between 1stJanuary and 30thAugust were collected. The prevalence of thrombocytopenia was 64.72 % and bleeding manifestation were recorded in 6.12% patients[97].

- In2011, Jayashree said that platelet can be used as a parameter to evaluvate the outcome of dengue fever. The major pathophysiological hallmark which determine disease severity and differentiate Dengue haemorrhagic fever from Dengue fever and other viral haemorrhagic fever is plasma leakage due to increased vascular permeability and abnormal homeostasis . Hypovolemic shock occurs as a consequence of and subsequent to, critical plasma volume loss .Abnormal hemostasis including increased capillary fragility, thrombocytopenia , impaired platelet function, consumptive coagulopathy and in the most severe form disseminated intravascular coagulation contribute to haemorrhagic manifestation [98].

- In 2011, Dongmei et al reported the kinetics of non-structural protein 1, IgM and IgG antibodies in dengue type 1 primary infection. A panel of 313 acute and early convalescent phase sera specimens from 140 DENV1 primary infected patients during an outbreak of dengue in Guangzhou, China, in 2006 were studied. Dengue NS1 presented high levels in acute-phase serum samples. It was

detectable as early as 1 day of illness and up to 14 days after onset. The sensitivity of NS1 detection was ranged from 81.8% to 91.1% with samples taken during the first 7 days. Anti-dengue IgM antibody was detectable on the third day of onset with the positive rate 42.9%, and rapidly increasing to 100% by day 8 of illness. Anti-dengue IgG antibody was detectable on the fifth day of onset with low level at the first week of onset, and slowly increasing to 100% by day 15 of illness. Combining the results of NS1 and IgM antibody detection allowed positive diagnosis in 96.9% -100% for samples taken after 3 days of onset .Dengue NS1 detection might shorten the window period by the first few days of illness . A combination of Dengue NS1 antigen and IgM antibody testing facilitates enhanced diagnostic rates. The procedures should be suitable for developing countries where dengue is endemic [99].

- In 2011 Zandi et al reported that four different types of bioflavanoid has antiviral activity against dengue virus type 2. Dengue is a major mosquito – borne disease currently with no effective antiviral or vaccine available. Effort to find antivirals for it has focussed on bioflavanoids, a plant derived polyphenolic compounds with many potential health benefits. In this study antiviral activity shown by four types of bioflavanoid against dengue virus -2 in vero cell is evaluvated. Anti-dengue activity of these bioflavanoid compounds were determined at various stages of DEN-2 infection and replication cycle. DENV replication was measured using Foci Forming Unit Reduction Assay (FFURA) and quantitative RT-PCR . Selectivity Index Value (SI) were determined as the ratio of cytotoxic concenteration 50 (IC50) for each compound. The half maximal inhibitory concenteration of quercetin against dengue virus was 35.7

microgram/ml when it is used after virus adsorption into the cells . The IC50 decreased to 28.9 microgram/ml when the cells were treated continuously for 5 hour before virus infection and up to 4 days post-infection. The SI values for quercetin were 7.07 and 8.74 microgram/ml respectively, the highest compared to all bioflavanoids studied. Naringin only exhibited anti adsorption effects against DENV2 with IC50 =168.2 microgram /ml and its related SI was 1.3. Daidzein showed a weak anti-dengue activity with IC50= 142.6 microgram/ml when the DENV 2 infected cells were treated after virus adsorption . The SI value for this compound was 1.03. Hesperetin did not show any antiviral activity against DENV2. The findings obtained from FFURA was corroborated by findings of the RT-PCR assays. Results from this study suggested that only quercetin had shown significant anti-DENV -2 inhibitory activities. Other bioflavanoids had demonstrated minimal to no significant inhibition of DENV-2 virus replication. These findings, together with those previously reported suggest that select group of bioflavonoids including quercetin and fisetin , exhibited significant inhibitory activities against dengue virus . This group of flavanoids , flavonol , may be investigated further to discover the common mechanisms of inhibition of dengue virus replication[100].

- In 2011, Blacksell etal diagnosed acute dengue infections using six commercial point-of care tests from 259 Sri Lankan patients with acute fevers (99 confirmed dengue cases and 160 patients with other confirmed acute febrile illness); 1) the Merlin dengue fever IgG and IgM combo device , 2) Standard diagnostics Dengue Duo non structural (NS1) antigen and IgG and IgM combo device , 3)the Biosynex dengue fever IgG and IgM assays ,4) Bio-rad NS1

antigen strip 5)Panbio Dengue Duo IgG /IgM cassette 6) Panbio dengueNS1 antigen strip. The median number of days of fever prior to admission sample collection was 5 days . Sensitivity and specificity of the NS1 antigen tests ranged from 49 to 50 % and from 93% to 99% respectively, and the sensitivity and specificity of the IgM antibody ranged from 71 to 80% and from 46 to 90% respectively. Combining NS1 antigen and IgM antibody results from the Standard Diagnostics Dengue Duo test gave the best compromise of sensitivity and specificity (93 and 89% respectively) and provided the best sensitivity in patients presenting at different times after the fever onset. The Merlin IgM/IgG antibody tests correctly classified 64% and 86% of the primary and secondary dengue infection cases respectively, and the Standard Diagnostics IgM /IgG antibody tests correctly classified 71% and 83% of the primary and secondary dengue infection cases, respectively . This study provides strong evidence of the value of combining dengue antigen –and –antibody –based test results in the rapid diagnostics test (RDT) format for the acute diagnosis dengue[101] .

- In 2011 , Anne etal did a retrospective study which showed that the sensitivity of the combined kit did not vary significantly between the serotypes and was not affected by the immune status or by the interval of time between onset of fever and sample collection. The aim of the study was to evaluate a combined dengue rapid test for detection of NS1 antigen and IgM/IgG antibodies . During the prospective study , 157 patients were hospitalised for suspicion of dengue fever were enrolled . In the hospital laboratory, the overall sensitivity , specificity , positive predictive value (PPV) and negative predictive valueof NS1 /IgM, IgG combination test was 87.5%, 83.9%, 95.6% and 59.1% respectively , whereas

they were 94.4% , 90% , 97.5% , 77.1% respectively in National Reference Laboratory at Institute Pasteur , Cambodia[102].

- In 2011 Kulkarni etal had done study showing the association between platelet count and NS1 antigen. Serum samples from clinically suspected dengue cases were tested for NS1 , IgM, IgG by immuochromatography –based test. Platelet counts were obtained for all the positive cases and 150 dengue seronegative cases of fever that served as controls. Test results of dengue specific parameters were compared against platelet counts. Of the 2104 samples tested, 320 were positive for one or more dengue parameters . Of the 320 , 95 were positive for NS1 only , 161 showed IgM only while 9 showed IgG only . More than one marker was detected in the remaining 55 samples . Thrombocytopenia was more consistently associated whenever NS1 was detected compared to antibody detection [1].

- In 2012 , Ruthven teal had evaluated the platelet count in dengue fever along with seasonal variation with seasonal variation of dengue infection .Blood samples were collected from 1549 patients experiencing a febrile illness, clinically consistent with dengue infections . Serological confirmation of dengue infection was done and platelet count was done in all serologically positive cases.294 cases were confirmed as serologically positive. The difference between serologically positive cases during different months was significant. Larger proportions of serologically positive cases were observed among adults. Outbreak co-incised mainly with the post monsoon period and was significantly higher[103].

- In 2012, Pan Teal reported that Granulate –binding protein 1 participates in cellular antiviral response to dengue virus . Quantitative RT-PCR and Western blot showed that the expression of mouse Gbp1 was regulated in DENV-infected RAW264.7 cells . The intracellular DENV loads were significantly higher in Gbp1 silenced cells compared with controls . The expression levels of selective anti-viral cytokines were decreased in Gbp1 RNA treated cells, while the transcription factor activity of NF-KB was impaired upon Gbp1 silencing during infection[104].

- In 2012, Hughes et al reported the manipulation dengue virus E protein epitomes reduces potential antibody –dependent enhancement. Reducing the cross reactivity in the envelope glycoprotein of DENV may be an approach to improve the quality of the anti-DENV immune response. Sera from mice immunised with wild type, fusion peptide -,or domain III – substitution containing vaccines enhanced heterologous DENV infection invitro , unlike sera from mice immunized with a vaccine containing a combination of fusion peptide and domain III substitution. Passive transfer of immune of immune sera from mice immunised with fusion peptide and domain III substitutions also reduced the development of severe DENV disease in AG129 mice when compared to mice receiving wild type immune sera[105].

- In 2012, Jayratne et al evaluvated the WHO revised criteria for classification of clinical disease severity in acute adult dengue infection. The WHO guidelines were revised recently to identify patients with severe dengue (SD) early. The study was proceeded to determine the usefulness of the warning signs in the new WHO –guidelines in predicting the SD and we have also attempted to define

other simple laboratory parameters that could be useful in predicting the SD. Clinical and laboratory parameters were recorded in 184 patients in 2011, with confirmed dengue viral infections, admitted to a medical ward in two tertiary care hospitals in Colombo, Sri Lanka. The presence of 5 or more dengue warning signals were significantly associated with the development of severe dengue. The AST levels were significantly higher in patients with abdominal pain associated with severe dengue. High AST levels were also significantly associated with severe dengue. Platelet counts <20,000 cells /mm^3 were again significantly associated with severe disease .The PCR was positive in 26/84 of the patients and the infecting serotype was found to be DEN1 in all 26 patients[106].

- In 2012, Alfonso etal reported phylogenetic relationship between dengue virus type 3 isolated in Brazil and Paraguay and global evolutionary divergence dynamics. Dengue is the most important mosquito –borne viral disease world wide. Dengue virus comprises four antigenically related viruses named dengue virus type 1 to 4. DEN-3 was re-introduced into the Americas in 1994 causing outbreaks in Nicaragua and Panama. DEN-3 was introduced in Brazil in 2000 and then spread to most of the Brazilian States , reaching the neighbouring country , Paraguay in 2002 . In this study , we have analysed the phylogenetic relationship of DEN-3 isolated in Brazil and Paraguay with viruses isolated worldwide . We have also analysed the evolutionary divergence dynamics of DENV3 viruses. The entire open reading frame (ORF) of thirteen DENV-3 isolated in Brazil (n= 9) within each genotype and Paraguay (n=4) were sequenced for phylogenetic analysis .DENV – 3 grouped into three main

genotypes (I,II,and III) .Several internal clades were found within each genotype that we called lineage and sub-lineage . Viruses included in this study belong to genotypeIII and grouped together with viruses isolated in the Americas within the lineage III. The Brazilian viruses were further segregated into two different sub-lineage, A and B, and the Paraguayan into the sub lineage B. All three genotypes showed internal grouping. The nucleotide divergence was in average 6.7% for genotypes, 2.7% for lineages and 1.5 % for sub lineages. Phylogenetic trees constructed with any of the protein gene sequence showed the same segregation of the DENV-3 in three genotypes. This study had shown that two groups of DEN-3 genotype III circulated in Brazil during 2002-2009, suggesting different events of introduction of the virus through different regions of the country . In Paraguay, only one group DENV3 genotype III was circulating that is very closely related to the Brazilian viruses of sublineage B Different degree of grouping can be observed for DENV3 and each group showed a characteristic evolutionary divergence. Finally, they observed that any protein gene sequence can be used to identify the virus genotype . In 2012 Jiang et al reported that the co-circulation of two genotypes of dengue virus serotype 3 seen in Guangzhou, China in 2009. Dengue is emerging as the most important mosquito borne viral disease in the world. In mainland China, sporadic and large outbreaks of dengue illness caused by the four serotypes of dengue virus have been well documented . Guangdong province is the major affected area in China, and DENV1 has dominantly circulated in this area for a long time. In this study, a family cluster of DENV3 infection in Guangzhou was described. Three cases were diagnosed as dengue fever based on clinical

manifestation, serological and RT-PCR assays . Two DENV3 strains were isolated in C6/36 and the complete genome sequences were determined. Phyogenetic analysis revealed that the new DENV-3 isolates from the family cluster were grouped within genotype III. Considering the fact that several DENV-3 strains within genotype V is also identified in Guangzhou in 2009, at least two genotypes of DENV-3 also co-circulated. Careful investigation and virological analysis is to be done in the future[22].

- In 2012, Satya etal did a study on atypical manifestations of dengue fever . A prospective hospital based observational study was conducted at hospitals of Kasturba Medical College in Manglore over a period of two years . 150 ELISA confirmed IgM –dengue sero-positive cases satisfying WHO criteria were examined clinically and laboratory data assessed till they got discharged from hospital after ruling out other causes of fever . Atypical manifestations in dengue fever were noted and analyzed . Most common symptoms noticed were myalgia , headache , rash , arthralgia , pain in the abdomen and nausea . More than half of the study group had one or the other atypical manifestation . Liver function test derangement was most often seen . Most common atypical manifestation was hepatitis found in 40% patients . Febrile diarrhoea, renal failure ,acalculous cholecystitis and conduction abnormalities of heart were among other common manifestations . Three patients died of multi-organ dysfunction, disseminated intravascular coagulation and shock . Platelet count did not correlate with the severity of bleeding. Overall recovery rate was good. Clinical vigilance for these manifestations is important for timely detection and management as some of them could be fatal [107].

- In 2013, Stuart D Blacksell said that dengue fever, dengue haemorrhagic fever and, dengue shock syndrome are tropical diseases that cause significant humanitarian and economic hardship . It is estimated that more than 2.5 billion people are at risk of infection and more than 100 countries have endemic dengue virus transmission. Laboratory tests are essential to provide an accurate diagnosis of dengue virus infection so that appropriate treatment and patient management may be administered [108]. In many dengue endemic settings, laboratory diagnostic resources are limited and simple rapid diagnostic tests (RDTs) provide opportunities for point-of-care diagnosis.

- In 2013, Saurabh et al studied the importance of platelet count and serological markers in diagnosing dengue infections with special reference to NS1 antigen . Serum samples from clinically suspected dengue cases were tested for NS1, IgM and IgG by immunochromatography based test . Platelet counts were obtained for all positive cases and dengue seronegative cases of fever served as controls . Test results of dengue – specific parameters were compared against platelet count. The result were subjected to statistical analysis. Of the 159 samples tested, 17 were positive for one or more dengue parameters . Of the 17, 5 were positive for NS1 only, 6 showed IgM only while 1 showed IgG only . More than one marker was detected in the remaining 5 samples . Thrombocytopenia was more consistently associated whenever NS1 was detected compared to antibody detection rate significantly [109].

- In 2013, Muhammad et al did a randomised control trial to find the effectiveness of platelet transfusion in dengue fever. Scientific data regarding effects of platelet transfusion on platelets transfusion on platelet count in dengue–related

thrombocytopenia is scanty. A single centre, randomised non-blinded trial was conducted on adult patients with dengue fever and platelet count less than 30,000/microlitre . Patients were randomised to treatment and control group. Treatment group received single donor platelets. Patients with post-transfusion platelet increment (PPI>/=10,000/ microlitre and /or corrected count increment (CCI) >/=5,000/microlitre 1 hour post transfusion were considered responders. Primary outcome was platelet count increments at 24 and 72 hours. 87 patients were enrolled, and 43 recieved platelet transfusion .Mean PPI and CCI at 1 hour post transfusion in the treatment group were 18,800 /microlitre and 7000/microlitre respectively. 22 patients in the treatment group were non-responders . Mean platelet increments at 24 and 72 higher in the treatment group as compared to control group. Responders showed significantly higher increments when compared to non-responders and the control group at 24 hours. Significant differences were found between non-reponders and the control group at 24 hours but not at 72 hours. Patients with lower baseline platelet count were more likely to be non responders. Platelet transfusion neither prevented development of severe bleeding nor shortened time to cessation of bleeding . Three severe transfusion reactions and two deaths occurred in treatment group. Platelet transfusion did not prevent development of severe bleeding and was associated with significant side effects . Therefore, platelet transfusion should not be routinely done in the management of dengue fever [110].

- In 2013, Tewari did a study in the clinical profile of dengue fever and use of platelets in four tertiary level hospitals of Delhi in the year 2009 . This is retrospective study based on the record of 230 patients admitted in 2009 in four

tertiary level hospitals situated at different locations in Delhi. 163 cases had dengue fever and 25 cases had evidence of haemorrhagic manifestation. The predominant presenting symptoms of dengue syndrome fever cases were that of vomiting, pain abdomen, headache, myalgia and nausea . Major bleeding manifestation were epistaxis, haematemesis and malena . Hepatomegaly with pleural effusion and ascites was the commonest finding. Severe thrombocytopenia as low as 2000 was observed in cases of dengue fever and another of DHF without any bleeding manifestation in DF and minor bleeding manifestations in DHF . Fresh blood has been given to one patient with of DF and two patients of DHF. There is significant difference in the value of p in the average number of days of stay in hospital for those who are given platelet transfusion. Two deaths have been reported in cases of DF –one due to atypical presentation, and other due to gastro intestinal bleeding[111].

- Vivaldo did a meta-analysis on the diagnostic accuracy of two commercial NS1 Antigen ELISA tests for early dengue virus detection. A total of 30 studies containing 12,105 total enrolled patients were present in the study. The results were as follows 1) Panbio assays showed low performance with sensitivity of 66% and specificity of 99%. Platelia assays showed higher performance with sensitivity of 74% and specificity of 99%. It was found that lowest sensitivity values were for secondary infections and was found mainly for DEN4. When it came to clinical manifestation the sensitivity of Platelia was found to be 69% for fever and dengue haemorrhagic fever. From the above it could be concluded that DENV1 gave a higher sensitivity for both of the tests where as DENV4 gave a lower sensitivity for the two tests. More over factors such as type of infection,

geographical origin of samples and viral serotypes which influences the tests require further investigation in order to optimize the diagnosis[112].

- Kwissa et al proposed that dengue virus infection induces expansion of a CD14(+) CD16(+) monocyte that stimulates plasma blast differentiation. In this study a systems biological approach was used to analyse immune responses to dengue in humans. Transcriptomic analysis of whole blood revealed that genes encoding proinflammatory mediators and type 1 interferon –related proteins were associated with high DENV levels during initial symptomatic disease. Additionally, CD 14(+) CD(+) monocytes increased in the blood. This study provided a detailed picture of innate responses to dengue and highlight a role for CD14(+) CD16(+) monocytes upregulated CD16 and mediated differentiation of resting B cells to plasmablasts as well as immunoglobulin and M secretion . These findings provide a detailed picture of innate responses to dengue and highlight a role for CD14(+) CD (+) monocytes in promoting plasma blast differentiation and anti-DENV antibody responses.[113]

- In 2014, Silva et al used a thiophene-modified screen printed electrode (SPE) for detection of the dengue virus non-structural protein (NS1) . A sulphur – containing heterocyclic compound (thiophene) was incorporated to a carbon ink to prepare prepare reproducible screen printed electrodes. After cured , the thiophene SPE was coated by gold nanoparticles conjugated to Protien A to form a nanostructured surface . The Anti-NS1 antibodies immobilized via their Fc portions via Protien A, leaving their antigen specific sites free circumventing the problem of a random antibodies immobilization .Amperometric responsesto the NS1protien of dengue virus were obtained by cyclicvoltammetries

performed in presence of ferrocyanide as redox probe. The calibration curve of immunosensor showed a linear response from 0.04 microgram/ Litre to 0.6 microgram/Litre of NS1 with good linear correlation .[114]

- Arshi etal in 2014 evaluvated the efficacy of various immuochromatographic rapid tests for dengue diagnosis . In this study sera from a large number of patients admitted to National Institute of Blood Diseases and Bone Marrow Transplantation (NIBD) were used to determine the efficacy of NS1 antigen , IgA, IgM basedrapid test devices for dengue diagnosis . The dengue NS1 antigen based device was least efficient while among the antibody based device the dengue IgA rapid test was comparatively better. In this study IgA RDT could be cost effective and efficient rapid test device for dengue diagnosis.[115]

- Selvaraj in 2014 did a comparative study between rapid immune chromatography and two enzyme-linked immunosorbent assay kits for NS1 antigen. In this test the sensitivity and specificity of newly introduced rapid combo kit against two conventional ELISA kits is assessed. The performance of this kit is quite satisfactory since excellent agreement of 94% was observed with particular reference to NS1 antigen detection among all three kits [116].

Materials & Methods

MATERIALS AND METHODS

The study group consists of all febrile patients with fever of duration less than 5 days attending the medical OPD for a period of one year from July 2013. The study was done after receiving permission from institutional ethical and research committee. A total of 176 blood samples from fever patients were received in the microbiology lab of central lab in our institution. Blood from the out patients was collected in the microbiology lab whereas from in patients were collected in the ward and then transported immediately to the lab .After the arrival of samples in lab an immunochromatographic test (card test) for NS1 antigen, IgM and IgG antibodies was done by using a kit, of SD BIO LINE purchased from Star laboratories, Nagercoil . This was followed by ELISA (Enzyme Linked Immunosorbent Assay) for detection of NS1, IgM and IgG antibodies of dengue virus. ELISA was done for all samples received in our lab .The ELISA kits were of Panbio purchased from Star Laboratories, Nagercoil. The other parameters such as age , gender , fever duration and platelet count were also recorded.

INCLUSION CRITERIA

1) Clinically suspected dengue fever cases

   Cases with fever, headache, retrobulbar pain, pain in the back and the limbs, lymphadenopathy, maculopapular rash and any haemorrhagic manifestations.

2) Patients with day 1 of fever, not possible to correctly fix in every patient.

EXCLUSION CRITERIA

1) Those who are unwilling to participate in this study.

2) Fever patients found to be positive for other tests such as Widal test ,malarial parasites etc .

IMMUNOCHROMATOGRAPHIC TEST (CARD TEST)

SD BIO LINE DENGUE DUO (DENGUE NS1 Ag COMBO)

This is a rapid invitro immunochromatographic, one step assay designed to detect both dengue virus NS1 antigen and differential IgM/IgG antibodies to dengue virus in human serum, plasma or whole blood. The test device contains two test devices;

1) Dengue NS1 antigen test on the left side

2) Dengue IgM/IgG antibodies on the right side

The dengue NS1 antigen rapid test in the left side is an in-vitro immunochromatographic one step assay designed for the qualitative determination of dengue virus NS1 antigen in human serum, plasma or whole blood. This test device contains a membrane strip, which is pre-coated with anti-dengue NS1 antigen capture on the test band. The dengue IgM/IgG rapid test on the right side is a solid phase immunochromatographic assay for detection of IgM and IgG antibodies to dengue virus in human serum, plasma or whole blood.

ACTIVE INDREDIENTS OF MAIN COMPONENTS

SD Bioline Dengue NS1 Ag ;

One test strip included; Gold conjugate as the main component: Mouse monoclonal anti-dengue NS1 –gold colloid (0.27 +/- 0.05 microgram)

Test line: Mouse monoclonal anti- dengue NS1 (0.72 +/- 0.14 microgram)

Control line: Goat anti-mouse IgG (0.72 +/-0.14 microgram)

SD BIOLINE DENGUE IgG /IgM

One test strip; Gold conjugate as the main component: Recombinant Dengue envelope protein – gold colloid (1+/- 0.2 micrograms)

Test line; Mouse monoclonal anti-human IgG (5+/- 1 microgram)

Control line; Rabbit anti-dengue IgG (2.5 +/- 0.5 micrograms)

PRINCIPLE [63, 112,113]

The dengue NS1 antigen test device result window has two pre-coated lines, **T** (NS1 antigen test line) and **C** (control line). Both the test line and the control line in result window are not visible before applying any samples. The control line is used for procedural control and should always appear if the test procedure is performed correctly. The anti-dengue NS1 antigen-colloid gold conjugate and serum, plasma or whole blood move along the membrane chromatographically to the test region (T) and forms a visible line as the antibody- antigen –antibody gold particle complex forms.

Dengue IgG/IgM test device has three precoated line, G ;(Dengue IgG test line), M (Dengue IgM test line) and C (control line) on the surface of the device. All the three lines are not visible before applying the sample. Control line should always appear if the test procedure is performed properly. A purple G or M lines will be visible in the result window if there are enough IgG and/ or IgM antibodies to the dengue virus in the sample. When a specimen is added to the sample well, anti-dengue IgGs and IgMs in the specimen will react with the recombinant dengue virus envelope proteins-colloidal gold conjugate and forms a complex of antibodies-

antigen. As this complex migrates along the length of the test device by capillary action, it will be captured by the relevant anti-human IgG and/ or anti-human IgM immobilised in two test lines across the test device and generate a coloured line.

MATERIALS REQUIRED FOR THE TEST

1) SD BIO-LINE Dengue Duo kit ; This kit contains the following items ;

A) Dengue NS1 antigen and Dengue IgM/IgG combo device

B) Assay diluents for dengue IgM/IgG test

C) 10 micro litre capillary pipette for IgM/IgG test

D) Disposable dropper for NS1 antigen test

E) A pair of gloves.

SPECIMEN COLLECTION;

Blood was collected by venipuncture after getting the written consent from the patient. Whole blood was collected into collection tube containing EDTA and then centrifuged at 3000 rpm for about 5-10 minutes to get the plasma which was then utilised for the card test.

KIT STORAGE

The kit was stored at a temperature less than 30 degree Celsius in a refrigerator and it was never freezed. The shelf life of the kit is indicated on the outer package and the tests were performed before the expiry date of the kit.

PROCEDURE

IMMUNOCHROMATOGRAPHIC TEST FOR Dengue NS1 ANTIGEN

1) Remove the test device from the foil pouch, and place it on a flat, dry surface.

2) With the help of a disposable dropper add 3 drops of specimen in to the sample well S.

3) Interpret the test result in 15to 20 minutes

4) The test was not interpreted after 20 minutes.

IMMUNOCHROMATOGRAPHIC TEST FOR IgM/IgG ANTIBODIES

1) With a 10 microlitre capillary pipette , add 10 microlitre of specimen into the square sample well S

2) Add 4 drops of assay diluents to the assay diluents well (round shaped)

3) Interpret the test result in 15-20 minutes

ENZYME LINKED IMMUNOSORBENT ASSAY(ELISA)

DENGUE EARLY ELISA (Panbio Dengue Early ELISA)

Dengue early ELISA is a dengue NS1antigen capture ELISA, which is used for qualitative detection of NS1 antigen in serum.

PRINCIPLE[10, 15]

Serum dengue NS1 antigen, when present, binds to anti-NS1 antibodies attached to the polystyrene surface of microwells. Residual serum is removed by washing and HRP conjugated Anti-NS1 Mab (Horse radish peroxidise conjugated Anti-NS1 monoclonal antibody) is added. After incubation, the microwells are

washed and a colourless substrate system, tetramethyl Benzidine /hydrogen peroxide (TMB chromogen) is added. The substrate is hydrolysed by the enzyme and the TMB changes to blue colour .After stopping the reaction with the acid, the TMB turns yellow. Colour development is indicative of the presence of dengue NS1 antigen in the test sample.

Materials required for Early Dengue ELISA

1) Anti-NS1 Antibody Coated Microwells (12x8 wells) ; microwells are coated anti-NS1 antibodies

2) HRP conjugated Anti-NS1 Mab (Horse radish peroxidise conjugated Anti-NS1 monoclonal antibody with preservative) ;one bottle 15 ml (orange colour). The preservative used is 0.1% Proclin.

3) Wash buffer; one bottle, 60ml of 20x concentrate of phosphate buffered saline (pH 7.2-7.6) with Tween20 and preservative. Dilute one part wash buffer with 19 parts of distilled water. Diluted buffer may be stored for one week at 2-25 ^{0}C. Crystallisation may occur at low temperatures which may be corrected by incubation at 37^{0}C until clear.

4) Sample diluent ; One bottle of sample diluent , 22 ml (brown colour). Ready for use. It consists of Tris buffered saline with preservative (o.1% Proclaim) and additives. The sample diluents remains stable at 2-8^{0}C until expiry.

5) TMB Chromogen; one bottle 15 ml ready for use. A mixture of 3, 3, 5, 5'-tetramethyl Benzidine and hydrogen peroxide in a citric acid citrate buffer. TMB chromogen is stable at 2-8^{0}C and can be used until expiry .

6) Positive control; one purple capped vial, 1.2 ml recombinant antigen

7) Calibrator ; Two orange –capped vials , 1.5 ml recombinant antigen

8) Negative control ; one white –capped vial , 1.2 ml human serum

The two controls and the calibrator are stored at 2-8^{0}C until expiry

9) Stop solution ; one red capped bottle , 15 ml ready for use, 1M phosphoric acid. Stop solution can be stored at 2-8^{0}C until expiry.

10) Adjustable micropipettors with disposable pipette tips (5-1000 microlitre capacity).

11) Deionised water.

12) Microplate washing system.

13) Micro plate reader as possible reader with 450 nm filter.

14) Timer.

15) Graduated cylinder.

16) Flask.

17) Test tubes

Specimen

The serum was separated as soon as blood was received in the lab and stored at 2-8 degree Celsius.

TEST PROCEDURE

1) All the reagents were equilibrated to room temperature before commencing the assay.

2) Required number of microwells was removed from the foil sachet.

3) Five microwells are required for positive control (P), Negative control (N) and Calibrator (CAL) in triplicate.

4) Using suitable test tubes or a microtitre plate, dilute the positive control, negative control, and calibrator and patient samples.

5) Add 75 microlitres of sample diluents to 75 microlitre of the sample and mix well.

6) Pipette out 100 microlitres of diluted test samples and controls into their respective micro wells.

7) Cover plate and incubate for one hour at 37 +/- 1^0C.

8) Wash six times with diluted wash buffer.

9) Pipette 100 microlitre HRP conjugated Anti-NS1 Mab into each well.

10) Cover the plate and incubate for one hour at 37 +/- 1^0C

11) Wash six times with diluted wash buffer.

12) Pipette 100 microlitre of TMB into each well.

13) Incubate at room temperature for 10 minutes. A blue colour will develop.

14) Pipette 100 microlitre of stop solution to all wells in the same sequence and timing as the TMB addition. The blue colour will change to yellow.

15) Within 30minutes the absorbance of each well is read at a wavelength of 450 nm with a reference filter of 600-650 nm.

QUALITY CONTROL

Each kit contains calibrator, positive and negative controls. Acceptable values for these were found on the accompanying specification sheet .The negative and the positive controls are intended to monitor for substantial reagent failure. The test was considered invalid and was repeated if the absorbance readings of either controls or the calibrator do not meet the specifications.

CALCULATIONS

1) The calibration factor is batch specific and was obtained from the specification sheet before commencing the calculations

2) Cut off value; it is the average absorbance of the calibrator triplicates multiplied by the calibration factor.

3) Index value ; Sample absorbance / Cut off value

4) ELISA units can be calculated by multiplying the index value by 10.

INTERPRETATION OF THE RESULTS

INDEX	ELISA UNITS (Panbio units)	RESULT
<O.9	<9	NEGATIVE
0.9-1.1	9-11	EQUIVOCAL
>1.1	>11	POSITIVE

RESULT	INTERPRETATION
Negative	No detectable dengue NS1 antigen
Equivocal	Equivocal samples were repeated
Positive	Presence of detectable dengue NS1 antigen

DENGUE IgM CAPTURE ELISA

The dengue IgM Capture ELISA is used for qualitative detection of IgM antibodies to dengue antigen in the serum.

PRINCIPLE [54,55,69]

Serum antibodies of the IgM class when present combines with anti-human IgM antibodies attached to the polystyrene surface of the microwell test strips. A concentrated pool of dengue 1-4 antigens is diluted to the correct working volume with antigen diluents. The antigens are produced using an insect cell expression system and immunopurified utilising a specific monoclonal antibody. An equal volume of the HRP Conjugated Monoclonal antibody (Mab) is added to the diluents antigen, which allows the formation of antigen- Mab complexes. Residual serum is removed from the assay plate by washing, and the complexed antigen –Mab is added to the assay plate. After incubation, the microwells are washed and a colourless substrate system, tetramethylbenzidne /hydrogen peroxide (TMB Chromogen) is added. The substrate is hydrolysed by the enzyme and the chromogen changes to blue colour. After stopping the reaction with the acid, the TMB becomes yellow .Colour development is indicative of the presence of anti-dengue IgM antibodies in the test sample.

MATERIALS REQUIRED FOR DENGUE IgM CAPTURE ELISA;

Materials provided in the kit are;

1) Anti-human coated microwells-(12x8); Microwells are coated with anti-human IgM antibodies

2) Dengue 1-4 Antigens (Recombinant); one clear capped vial, 150 microlitres (blue), concentrated dengue viral antigens 1, 2, 3, 4 . Unused diluted antigen must be discarded. Concenterated antigen is stored at 2-8^{0}C until expiry .

3) Wash buffer; one bottle , 60ml of 20x concentrate of phosphate buffered saline (pH 7.2-7.6)with Tween 20 and preservative (0.1% Proclin) Crystallisation may occur at low temperatures . To correct , wash buffer is incubated at 37^{0}C until clear . Dilute one part wash buffer with 19 parts of distilled water . Diluted buffer may be stored for one week at 2-25 ^{0}C.

4) Sample Diluents; Two bottles, 50ml (pink) ready-to use. Tris buffered saline . (Ph; 7.2-7.6) with preservative (0.1% Proclin).and additives . It is stable at 2-8^{0}C until expiry.

5) Antigen Diluents ; One bottle , 50ml (clear) . Ready for use , Phosphate buffer containing preservative (0.1% Proclin and 0.005% gentamycin). It is stored at 2-8^{0}C until expiry.

6) HRP Conjugated Monoclonal Antibody Tracer; one bottle 7 ml (yellow) . Ready for use . Horse radish peroxidise conjugated monoclonal antibody tracer. . Stable at 2-8^{0}C until expiry with added protein stabilisers and preservative (0.1% Proclin).

7) TMB Chromogen (Tetramethyl benzidine chromogen);one bottle 15 ml ready for. It is a mixture of 3,3', 5,5' -tetramethylbenzidine and hydrogen peroxide in a citric acid citrate buffer (Ph 3.5-3.8) . Stable at 2-8 ^{0}C until expiry.

8) Positive control; One Black capped vial containing 200 microlitre human serum (containing 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8 ^{0}C until expiry.

9) Calibrator ; One orange – capped vial , 400 microlitre human serum (containing o.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8 ^{0}C until expiry.

10) Negative control; One white capped vial , 200 microlitre of human serum (contains 0.1% sodium azide and 0.005% gentamicin sulphate). Stable at 2-8 ^{0}C.

11) Stop solution; One red capped bottle , 15 ml , Ready for use (1M phosphoric acid). Stable at 2-25 ^{0}C until expiry.

Additional materials required are ;

12) Accurate adjustable micropipettors with disposable pipette tips (5-1000 microlitre capacity)

13) Deionised water.

14) Microplate washing system.

15) Microplate reader with 450nm filter.

16) Timer.

17) Graduated cylinder.

18) Flask.

19) Test tubes

20) Glass or plastic tubes or vials .

PROCEDURE

Before commencing the test procedure , ensure all the reagents are equilibrated to the room temperature.

1) Remove the required number of microwells from the foil sachet . Five microwellsare required for Negative control (N), Positive control (P), and Calibrator (CAL) in triplicate. Remaining unused microwells were sealed tightly in the foil sachet.

2) Using test tubes positive control, negative control , calibrator and patient serum were diluted .

3) Serum Dilution; To 10 microlitre serum add 1000 microlitre of sample diluents and mix well.

4) Antigen was diluted 1/250 using the antigen diluents. As per the kit , 10 microlitre of the antigen was diluted into 2.5 ml of antigen diluents. A volume of 2.5 ml of diluted antigen is required strip. The remaining unused concentrated antigen was stored at a temperature of 2-8 degree Celsius.

5) Required volume of diluted antigen was mixed with an equal volume of Mab Tracer in a clean vial , gently mixed and left at room temperature .

6) Assay plate; Within 10 minutes after mixing the Mab tracer and diluted antigen, pipette 100 microltre diluted patient sample and controls into their respective microwells of the assay plate .

7) Cover the plate and incubate for 1 hour at 37^0C.

8) Wash six times with diluted wash buffer.

9) Antigen-MAb tracer solution was mixed and 100 microlitre of antigen-MAb complex was transferred from antigen vial to the appropriate wells of the assay plate.

10) The plate was covered with a foil paper and incubated for 37^0C.

11) The wells were washed six times with diluted wash buffer .

12) 100 microlitre of TMB was transferred into each well and then incubated for 10 minutes at room temperature . A blue colour will develop .

13) Pipette out 100 microlitre of stop solution into all wells in the same sequence and timing as the TMB solution and mixed well . Then blue colour will change to yellow colour.

14) The absorbance of each well at a wavelength of 450 nm is read.

QUALITY CONTROL

Each kit contains calibrator, positive and negative controls. Acceptable values for these were found on the accompanying specification sheet .The negative and the positive controls are intended to monitor for substantial reagent failure. The test was considered invalid and was repeated if the absorbance readings of either controls or the calibrator do not meet the specifications.

CALCULATIONS

1) The calibration factor is batch specific and was obtained from the specification sheet before commencing the calculations

2) Cut off value; it is the average absorbance of the calibrator triplicates multiplied by the calibration factor .

3) Index value ; Sample absorbance / Cut off value

4) ELISA units can be calculated by multiplying the index value by 10.

INTERPRETATION OF THE RESULTS

INDEX	ELISA UNITS(PANBIO UNITS)	RESULT
<O.9	<9	NEGATIVE
0.9-1.1	9-11	EQUIVOCAL
>1.1	>11	POSITIVE

RESULT	INTERPRETATION
Negative	No detectable dengue IgM antibody
Equivocal	Equivocal samples were repeated
Positive	Presence of detectable dengue IgM antibody.

DENGUE IgG CAPTURE ELISA [56,60]

The Dengue IgG ELISA is used for the qualitative presumptive detection of elevated IgG antibodies to dengue virus in patients with secondary infection

PRINCIPLE

Serum antibodies of the IgG class when present combines with anti-human IgM antibodies attached to the polystyrene surface of the microwell test strips . A concentrated pool of dengue 1-4 antigens is diluted to the correct working volume with antigen diluents. The antigens are produced using an insect cell expression system and immunopurified utilising a specific monoclonal antibody . An equal volume of the HRP Conjugated Monoclonal antibody (Mab)is added to the diluents antigen, which allows the formation of antigen- Mab complexes . Residual serum is removed from the assay plate by washing , and the complexed antigen –Mab is added

to the assay plate. After incubation , the microwells are washed and a colourless substrate system , tetramethylbenzidne /hydrogen peroxide (TMB Chromogen)is added . The substrate is hydrolysed by the enzyme and the chromogen changes to blue colour . After stopping the reaction with the acid , the TMB becomes yellow .Colour development is indicative of the presence of anti-dengue IgG antibodies in the test sample .

MATERIALS REQUIRED FOR Dengue IgG CAPTURE ELISA

Materials provided in the kit are;

1) Anti-human coated microwells-(12x8); Microwells are coated with anti-human IgG antibodies

2) Dengue 1-4 Antigens (Recombinant); one clear capped vial , 150 microlitre (red), concentrated dengue viral antigens 1, 2, 3, 4. Unused diluted antigen must be discarded. Concenterated antigen is stored at 2-8^{0}C until expiry.

3) Wash buffer; one bottle, 60ml of 20x concentrate of phosphate buffered saline (pH 7.2-7.6)with Tween 20 and preservative (0.1% Proclin) The wash buffer is to be diluted, that is one part of wash buffer with 19 parts of distilled water . Diluted buffer may be stored at 2-25 ^{0}C for one week.

4) Sample Diluent; Two bottles, 50ml (pink) ready-to use. Tris buffered saline pH7.2-7.6 with added preservative (0.1% Proclin) and additives. Stable at 2-8^{0}C until expiry.

5) Antigen Diluent; One bottle, 50ml (clear) . Ready for use, Phosphate buffer containing preservative (0.1% Proclin and 0.005% gentamycin) . It is stored at 2-8 ^{0}C until expiry.

6) HRP Conjugated Monoclonal Antibody Tracer; one bottle 7 ml (green) . Ready for use. Horse radish peroxidise conjugated monoclonal antibody tracer with added preservatives and protein stabilisers. Stable at 2-8^{0}C until expiry.

7) TMB Chromogen (Tetramethyl benzidine chromogen);one bottle 15 ml ready for use . A mixture of 3, 3',5,5'-tetramethyl benzidine and hydrogen peroxide in a citric acid citrate buffer. Stable at 2-8^{0}C until expiry.

8) Reactive control ; One red capped vial containing 200 microlitre human serum (containing 0.1% sodium azide and .005% gentamycin sulphate). Stable at 2-8^{0}C until expiry.

9) Calibrator; One yellow – capped vial, 400 microlitre human serum (containing 0.1% sodium azide sulphate). Stable at 2-8^{0}C until expiry.

10) Negative control; one green capped vial, 200 microlitre human serum (contains 0.1% sodium azide and .005% gentamycin sulphate). Stored at 2-8 ^{0}C until expiry.

11) Stop solution; One red capped bottle, 15 ml, Ready for use (1M phosphoric acid). Stable at 2- 25 ^{0}C until expiry.

Additional materials required are ;

12) Accurate adjustable micropipettors with disposable pipette tips (5-1000 microlitre capacity)

13) Deionised water.

14) Microplate washing system.

15) Microplate reader with 450nm filter.

16) Timer.

17) Graduated cylinder.

18) Flask.

19) Test tubes

20) Glass or plastic tubes or vials .

PROCEDURE

Before commencing the test procedure, ensure all the reagents are equilibrated to the room temperature.

1) Remove the required number of microwells from the foil sachet. Five microwells are required for Negative control (N), Positive control (P), and Calibrator (CAL) in triplicate. Remaining unused microwells were sealed tightly in the foil sachet.

2) Using test tubes positive control, negative control, calibrator and patient serum were diluted.

3) Serum Dilution; To 10 microlitre serum add 1000 microlitre of sample diluents and mix well.

4) Antigen was diluted 1/250 using the antigen diluents. As per the kit , 10 microlitre of the antigen was diluted into 2.5 ml of antigen diluents. A volume of 2.5 ml of diluted antigen is required strip. The remaining unused concentrated antigen was stored at a temperature of 2-8^{0}C.

5) Required volume of diluted antigen was mixed with an equal volume of Mab Tracer in a clean vial, gently mixed and left room temperature .

6) Assay plate; Within 10 minutes after mixing the Mab tracer and diluted antigen, pipette 100 microltre diluted patient sample and controls into their respective microwells of the assay plate .

7) Cover the plate and incubate for 1 hour at 37^0C.

8) Wash six times with diluted wash buffer.

9) Antigen-MAb tracer solution was mixed and 100 microlitre of antigen-MAb complex was transferred from antigen vial to the appropriate wells of the assay plate.

10) The plate was covered with a foil paper and incubated for 37 degree Celsius

11) The wells were washed six times with diluted wash buffer

12) 100 microlitre of TMB was transferred into each well and then incubated for 10 minutes at room temperature . A blue colour will develop .

13) Pipette out 100 microlitre of stop solution into all wells in the same sequence and timing as the TMB solution and mixed well . Then blue colour will change to yellow colour.

14) The absorbance of each well at a wavelength of 450 nm is read.

QUALITY CONTROL

Each kit contains calibrator , positive and negative controls. Acceptable values for these were found on the accompanying specification sheet .The negative and the positive controls are intended to monitor for substantial reagent failure . The test was considered invalid and was repeated if the absorbance readings of either controls or the calibrator do not meet the specifications.

CALCULATIONS

The calibration factor is batch specific and was obtained from the specification sheet before commencing the calculations

Cut off value; it is the average absorbance of the calibrator triplicates multiplied by the calibration factor.

Index value; Sample absorbance / Cut off value

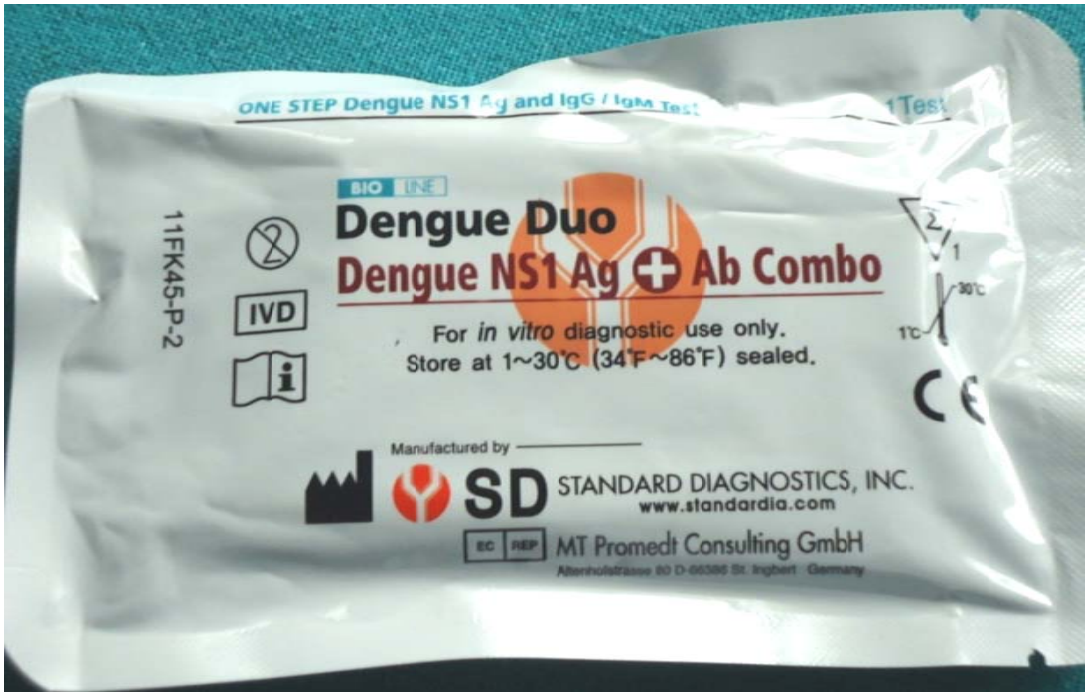
ELISA units can be calculated by multiplying the index value by 10.

INTERPRETATION OF RESULTS

INDEX	ELISA UNITS(Panbio units)	RESULT
<1.8	<18	NEGATIVE
1.8-2.2	18-22	EQUIVOCAL
>2.2	>22	POSITIVE

RESULT	INTERPRETATION
Negative	No detectable dengue IgG antibody
Equivocal	Equivocal samples were repeated
Positive	Presence of detectable dengue IgG antibody.

DENGUE NS1 Ag +Ab Combo (IMMUNO CHROMATOGRAPHIC TEST)



DENGUE NS1 Ag +Ab Combo (IMMUNO CHROMATOGRAPHIC TEST)(KIT OPENED)

DENGUE NS1 Ag +Ab Combo (IMMUNO CHROMATOGRAPHIC TEST)(KIT SHOWING POSITIVE TEST FOR NS1 Ag)

REAGENTS USED IN NS1 ANTIGEN ELISA

REAGENTS USED IN IgM ELISA

REAGENTS USED IN IgG ELISA

Results

RESULTS

The study was done using multi variant regression analysis with dependent binary variables. This model also assumes that *Z* is linearly related to the predictors.[114,115]

$$z_i = b_0 + b_1 x_{i1} + b_2 x_{i2} + \ldots + b_p x_{ip}$$

Where

x_{ij} is the j^{th} predictor for the i^{th} case

b_j is the j^{th} coefficient

p is the number of predictors

Z is a binary variable which takes value 1 for positive sample and zero for negative sample

Table1 **NS1 ANTIGEN CARD TEST**

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1(a)	platelet	.0001	.000	30.337	1	.0001*	1.000
	age	.012	.030	.154	1	.695	1.012
	Gender	-.280	.626	.200	1	.655	.756
	days	-1.476	.328	20.272	1	.0001*	.228
	Constant	7.719	1.913	16.285	1	.000*	2250.847

A Variable(s) entered on step 1: platelet, age, Gender, days

- P value of platelet count is 0.0001 . Since p value is less than 0.05 , association between platelet count and NS1 antigen is significant.
- P value of fever duration is less than 0.05 There is association between fever duration and NS1 antigen..

Table2; **IgM CARD TEST**

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1(a)	platelet	.000	.000	5.606	1	.018*	1.000
	age	.089	.045	3.900	1	.048*	1.093
	Gender	.074	.869	.007	1	.932	1.077
	days	.868	.384	5.108	1	.024*	2.383
	Constant	-6.856	2.509	7.467	1	.006	.001

A Variable(s) entered on step 1: platelet, age, Gender, days.

- The p value for platelet count, age and duration of fever is less than 0.05% . Hence there is association between these three parameters and IgM card test.

Table3; **NS1 ANTIGEN ELISA (PANBO UNITS)**

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1(a)	Platelet	.000	.000	30.337	1	.0001*	1.000
	Age	.012	.030	.154	1	.695	1.012
	Gender	-.280	.626	.200	1	.655	.756
	Days	-1.476	.328	20.272	1	.0001*	.228
	Constant	7.719	1.913	16.285	1	.000*	2250.847

A Variable(s) entered on step 1: platelet, age, and Gender days of fever

The p value of platelet count and duration of fever is less than 0.05 % . Hence there is association between NS1 ELISA and platelet and fever duration .

Table4; **IgM ELISA (PANBI-O UNITS)**

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1(a)	Platelet	.000	.000	7.281	1	.007*	1.000
	Age	.020	.020	.963	1	.327	1.020
	Gender	.275	.392	.491	1	.484	1.316
	Days	.598	.179	11.138	1	.001*	1.818
	Constant	-2.903	1.098	6.986	1	.008*	.055

A Variable(s) entered on step 1: platelet, age, Gender, days.

The p value of platelet count , duration of fever is less than 0.05% . Hence there is association between IgM ELISA and the above two parameters.

Table5 **IgG ELISA (PANBIO- UNITS)**

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1(a)	Platelet	.000	.000	4.012	1	.045*	1.000
	Age	-.030	.041	.551	1	.458	.970
	Gender	-.198	.819	.059	1	.809	.820
	Days	.301	.398	.572	1	.449	1.351
	Constant	-7.213	3.239	4.959	1	.026*	.001

A Variable(s) entered on step 1: platelet, age, Gender, days.

Here the p value of platelet count is only less than 0.05%. platelet count is associated with IgG ELISA.

Table 6, NS1 ANTIGEN CARD TEST

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Negative	119	67.6	67.6	67.6
	Positive	57	32.4	32.4	100.0
	Total	176	100.0	100.0	

The above table shows the frequency of NS1 antigen immunochromatographic test . The frequency of NS1 antigen immunochromatographic positive samples is 57 (32.4%) and the frequency of NS1 antigen immunochromatographic negative samples is 119 (67.6%) (Fig;4)

Table 7 , IgM CARD TEST

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Negative	169	96.0	96.0	96.0
	Positive	7	4.0	4.0	100.0
	Total	176	100.0	100.0	

The above table shows the frequency of IgM card test . The frequency of IgM card positive samples is 7 (4%) and the frequency of IgM card negative samples is 169 (96%). (Fig;5)

Table 8, IgG CARD TEST

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Negative	176	100.0	100.0	100.0

Table 9, NS1 ANTIGEN ELISA PANBI-O UNITS

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Negative	119	67.6	67.6	67.6
	Positive	57	32.4	32.4	100.0
	Total	176	100.0	100.0	

The above table shows the frequency of NS1 antigen ELISA samples. The frequency of NS1 antigen ELISA positive samples is 57 (32.4%) and the frequency of NS1 antigen ELISA negative samples is 119 (67.6%).(Fig 6)

Table 10; IgM ELISA PANBIO UNITS

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Negative	139	79.0	79.0	79.0
	Positive	37	21.0	21.0	100.0
	Total	176	100.0	100.0	

The above figure shows the frequency of IgM ELISA . The frequency of IgM ELISA positive samples is 37 (21%) and the frequency of IgM ELISA negative samples is 139(79%) (.Fig 7)

Table 11 ; **IgG ELISA PANBIO UNITS**

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Negative	168	95.5	96.0	96.0
	Positive	7	4.0	4.0	100.0
	Total	175	99.4	100.0	
Missing	System	1	.6		
Total		176	100.0		

The above figure shows the frequency of IgG ELISA . The frequency of IgG ELISA positive samples is 7 (4%) and the frequency of IgG ELISA negative samples is 168 (95.5%).(Fig 8)

Fig. 1

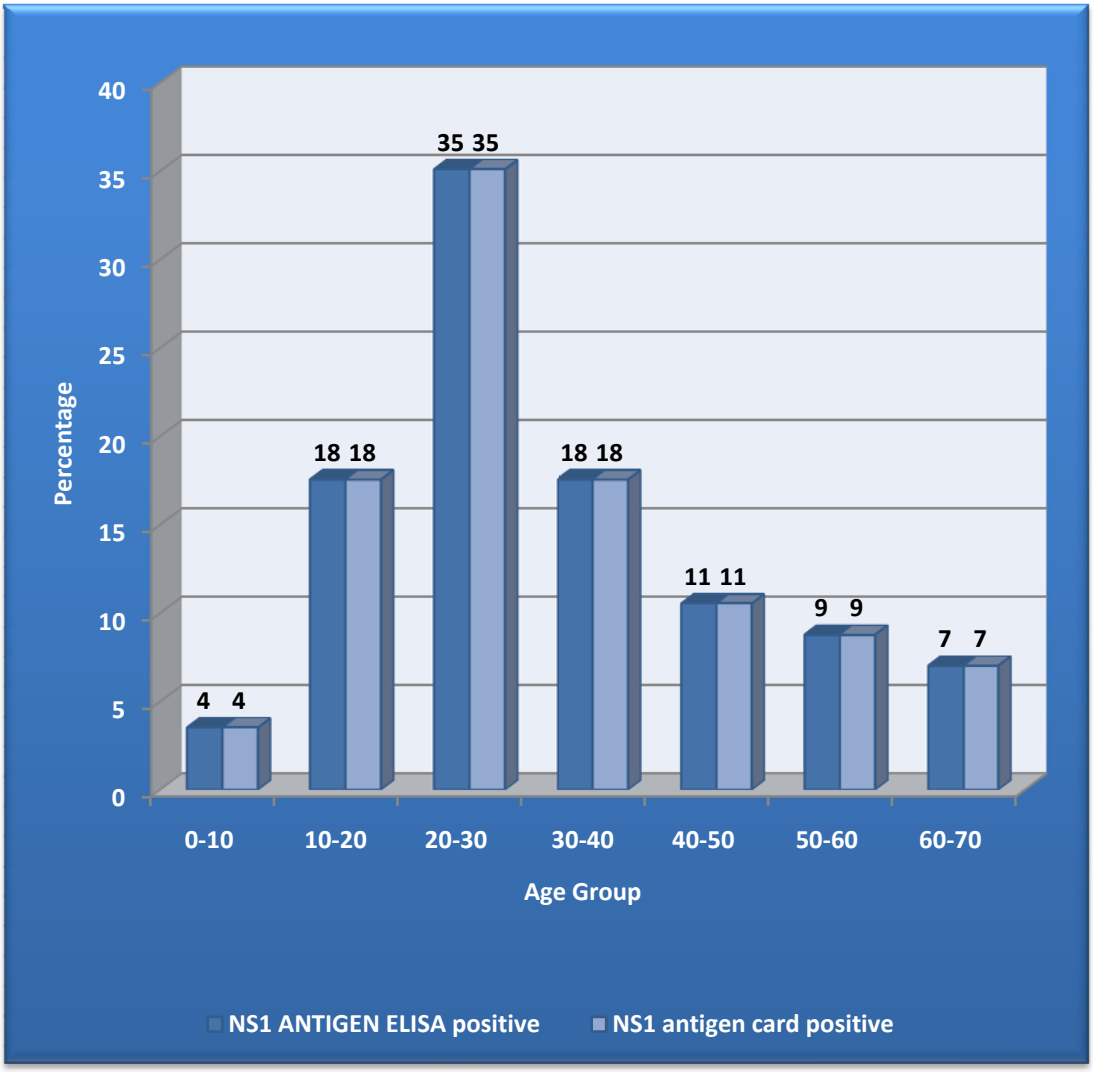
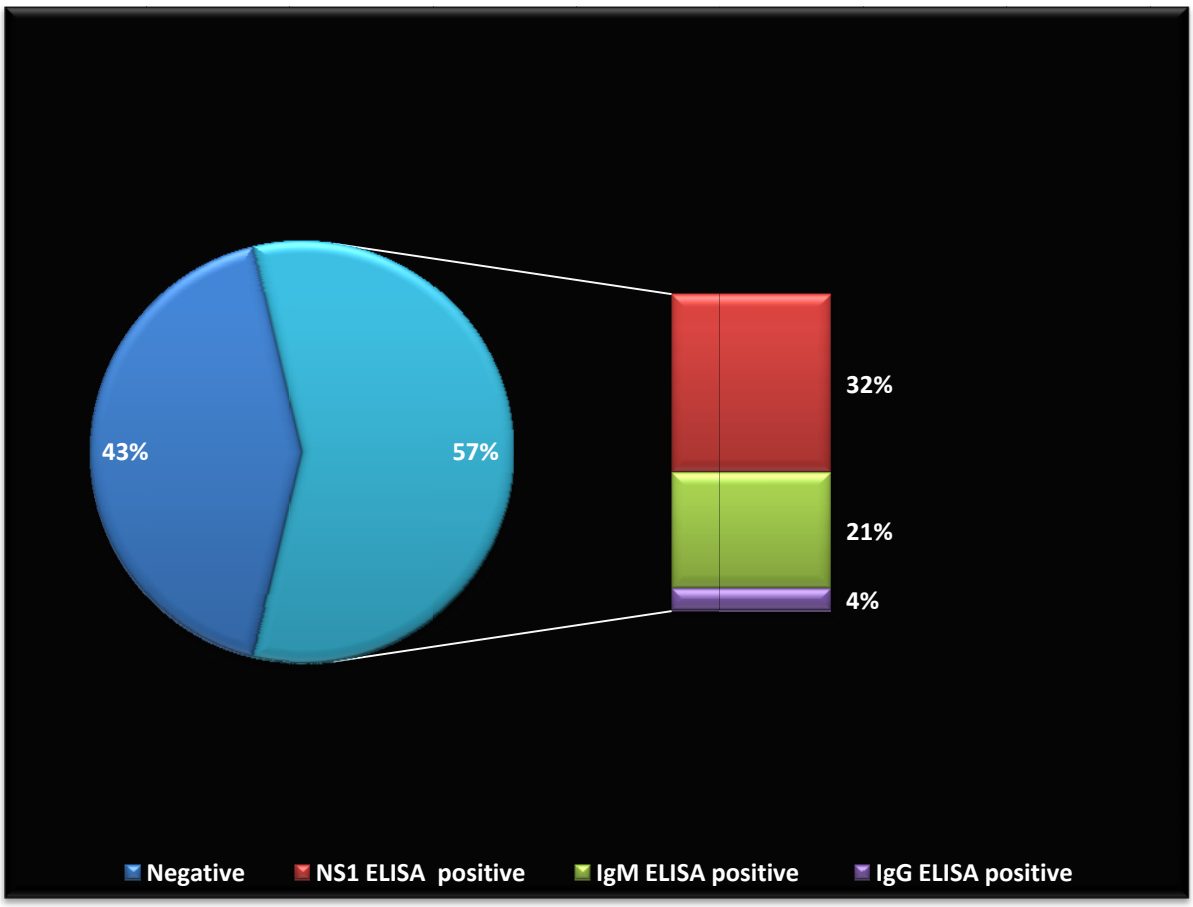


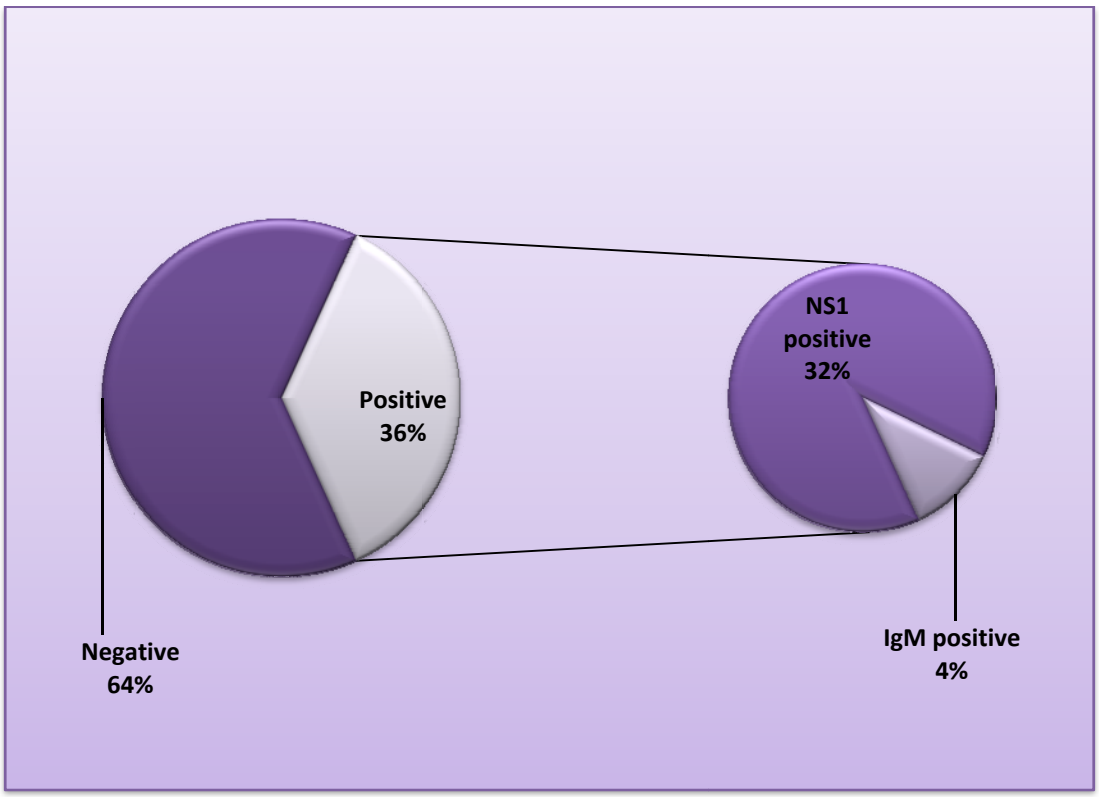
Fig 1 The above figure demonstrates the age wise percentage distribution of NS1 antigen positive cases.The NS1 positivity is maximum for the age group 20-30 years .

Fig2. PIE CHART FOR ELISA TEST



From the above figure it could be shown that 57% of the total sample were positive for one of the dengue ELISA(NS1 antigen, IgM/IgG antibody). Out of the 57% , 32% were positive for NS1 ELISA, 21% for IgM ELISA and 4% for IgG ELISA.

Fig 3, PIE CHART FOR ICT (CARD TEST)



In the above figure , 36% of the samples were positive for immunochromatographic test, out of which 32% were positive for NS1 antigen and only 4% were positive for dengue antibodies.

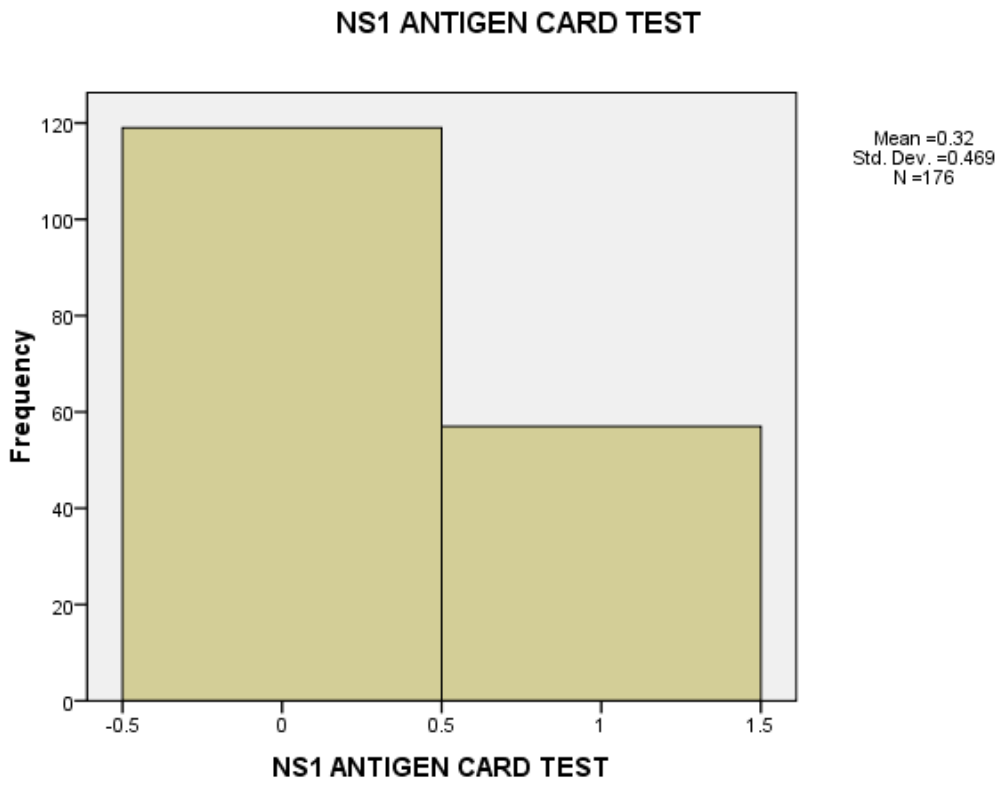


Fig 4, The above figure shows the frequency chart for NS1 antigen immunochromatographic test. The frequency of NS1 antigen card negative samples is found to be 119 (67.6%) and the frequency of positive samples is 57(32.4%) (Table 6)

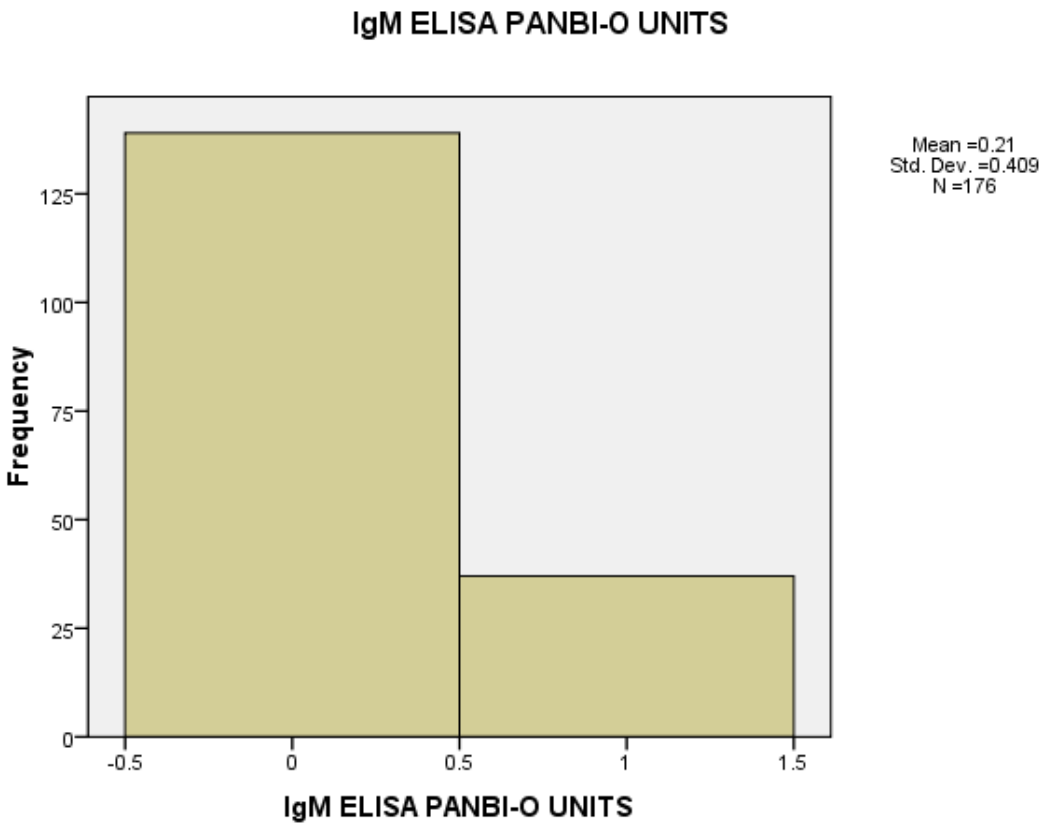


Fig5, The above figure shows the frequency distribution of IgM immunochromatographic test. The frequency of IgM card negative samples is 169(96%) and the frequency of IgM card positive samples is 7 (4%). [Table7]

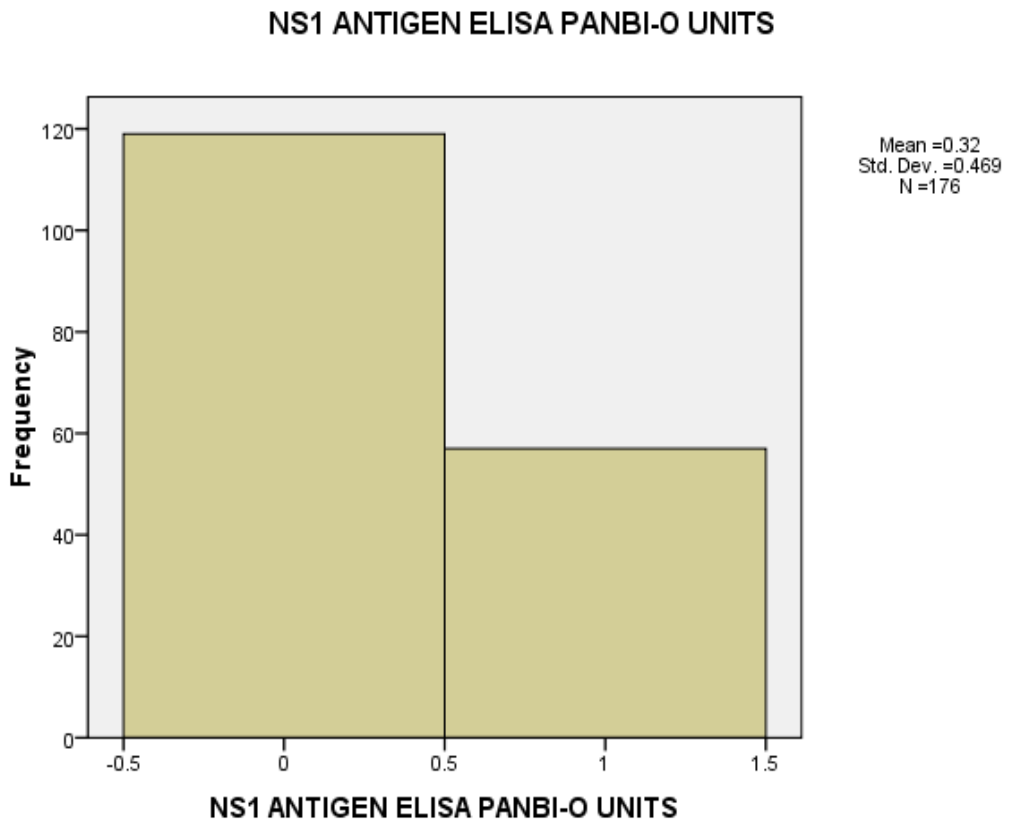


Fig 6, The above figure shows the frequency chart for NS1 antigen ELISA. The frequency of NS1 antigen ELISA negative samples is 119 (67.6%) and the frequency of NS1 antigen ELISA positive samples is 57(32.4%). [Table 9]

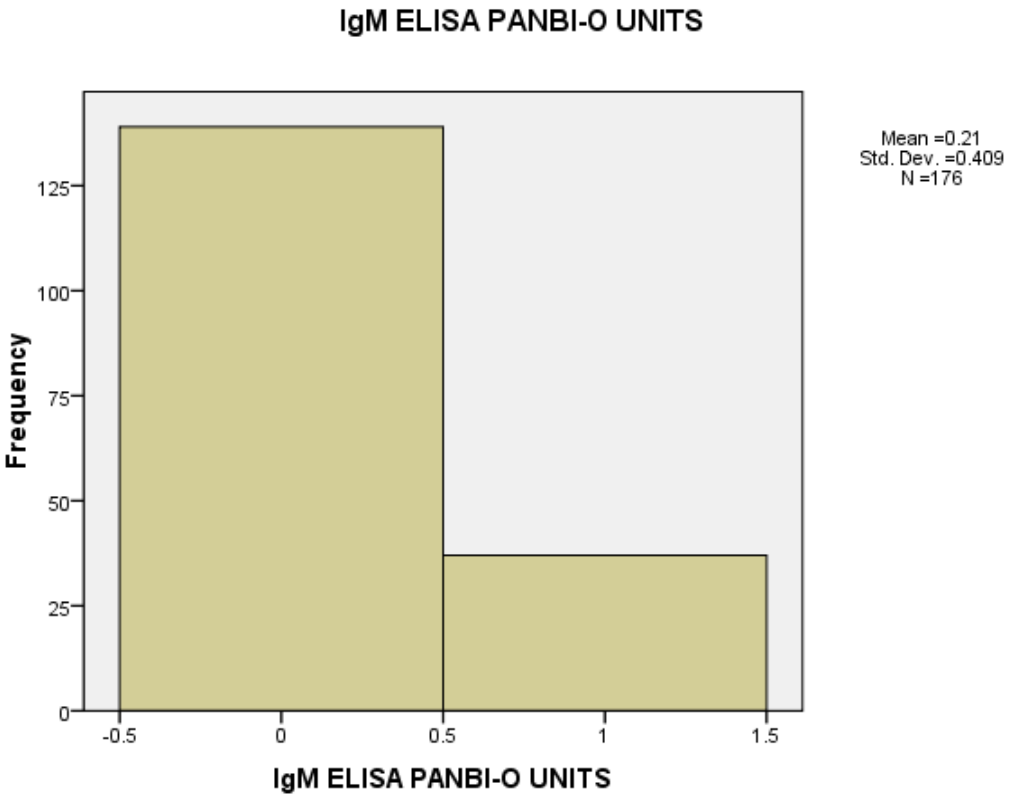


Fig 7, The above figure shows the frequency chart for IgM ELISA . The frequency of IgM ELISA positive samples is 37 (21%) and the frequency of IgM ELISA negative samples is 139 (79%) .[Table 10]

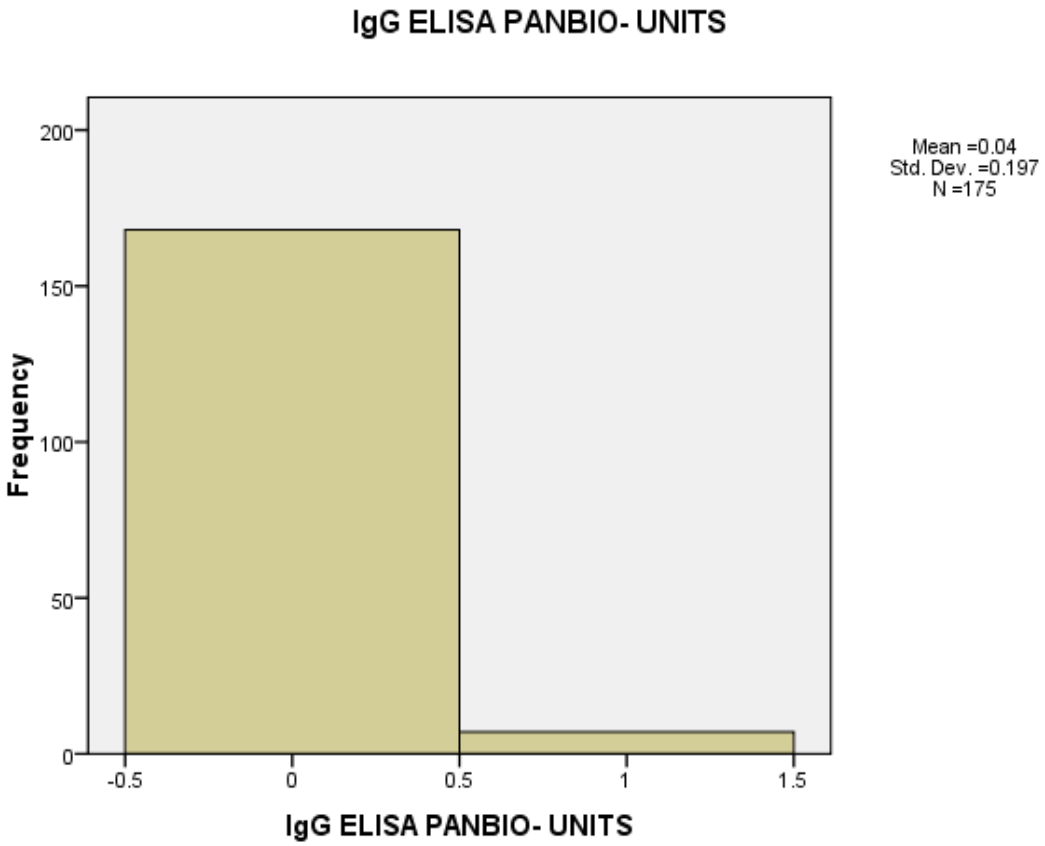


Fig 8, The above figure shows the frequency chart for IgG ELISA . The frequency of IgG ELISA negative samples is 168(95.5%) and the frequency of IgG ELISA positive samples is 7 (4%). [Table 11]

Discussion

DISCUSSION

Dengue fever is an important mosquito borne viral disease of humans . The disease is now present in more than hundred countries in Africa , America , Eastern Mediterranean , Western Pacific , and particularly in South East Asia. approaches had been applied for diagnosing dengue virus infection[22] . These methods include detection of the virus (by cell culture, immunofluorescence), detection of virus antigen (by enzyme linked immunosorbent assay, ELISA), detection of anti-dengue virus antibody [by haemagglutination Inhibition, (HI), complement fixation (CF), neutralisation tests, ELISA] and detection of viral nucleic acid by reverse transcriptase polymerase chain rection. However for confirmed dengue diagnosis dengue virus should be identified by isolation or nucleic acid detection or there should be a fourfold rise in antibody titre in paired sera in patients presenting with signs and symptoms consistent with dengue virus infection[102] .

The study was conducted at Sree Mookambika Institute of Medical Science , Kulasekharam for one year from July 2013. Blood samples from 176 clinically suspected cases of dengue infection (according to WHO criteria) with fever of duration less than 5 days were taken after receiving a written consent from the patient . Out of total 176 serum samples, 101 were positive for dengue seromarkers . The dengue seromarkers used in this study were NS1antigen , IgM and IgG antibodies. Two methods were employed in the study; a Rapid immunochromatographic test (card test) and ELISA . The tests were done for each of the dengue seromarkers. In addition to these specific markers, age gender, fever duration and platelet count were recorded for each patient.

Table 12. Comparison of efficacy of various dengue specific seromarkers in the diagnosis of dengue infection

DENGUE SEROMARKERS	CARD TEST POSITIVE	ELISA POSITIVE
NS1antigen	57(32%)	57(32%)
IgM antibody	7(4%)	37(21%)
IgG antibody	0	7(4%)

The distribution of various specific dengue parameters is shown in table 12. Out of 176 cases, 57 (32%) were positive only for NS1 antigen [Table 6&9]. Considering a very high sensitivity of NS1 it can be stated that , the diagnosis in 32% of cases would have been missed if NS1 was not included in the test panel. Kulkarni (2011) , Shrivastva(2011) , Datta(2010) have shown that NS1 was positive in 95 out of 320 cases (30%), 15 out of 91 cases (16%) and 140 out of 600 cases (23%) respectively in their studies[1] . From the above it can be said that a large number of cases would be missed if NS1 antigen is not included in the test panel for dengue virus infection detection. Those who are exclusively NS1 positives could be offered appropriate supportive therapy, thus avoiding any irrational use of antibiotics . The patients and their attendants could be briefed regarding the basics of vector biology and provided with mosquito nets during viraemic phase to further prevent the spread of infection to others. The dengue specific antibodies appear only by third to fifth day of fever in primary dengue infection[2] . Even in most secondary infections, both IgM and IgG type antibodies cannot be recorded before the third day. Both in primary and secondary dengue infection there is always a window period, both in primary when

only antibodies are detected. Moreover the utility of antibody relies mainly on the rising titres especially in endemic areas. The seromarker NS1 antigen which is a highly sensitive dengue marker for dengue infections is detected from the first day of fever . Among the 57 NS1 antigen positive cases 28 (49%) were detected on the second day of fever . This is in accordance with a study conducted by Hang etal (2008). The study reported the diagnostic accuracy of NS1 ELISA and lateral flow rapid tests for dengue sensitivity, specificity and relationship to viraemia and antibody responses. According to the study the sensitivity of both ELISA and NS1 – Lateral flow rapid tests were influenced by the patients duration of fever before entering the study. The tests were significantly more sensitive when the test samples were collected within 3 days of illness onset. If NS1 is positive, the right intervention could be initiated.

Following the initial infection, the dengue virus replicates to high titres in the blood before the patient becoming symptomatic enough to go to a physician. During the viraemic phase of dengue infection, NS1 antigen are produced during virus replication in either membrane associated or secreted forms. Soluble NS1 circulates in the serum of patients of these patients and thus provides an excellent tool for diagnosis of acute dengue infections[11].

In this study ELISA is taken as the gold standard for serological testing of dengue fever. From the table 6, it could be shown that , NS1antigen was positive in 57 / 176(32%) cases by immunochromatographic test. These 57 cases (32%) were also found to be positive for ELISA and those cases which were found to be negative by card test were found to be negative by ELISA. Thus it could be shown that the rapid immunochromatographic test used in this study is equally sensitive and specific to

ELISA. Although virus isolation is considered to be the gold standard for laboratory diagnosis of dengue viral infections , it involves large labour and requires at least 6-10 days for virus to replicate[1,15] . On the other hand RT-PCR is expensive and require sophisticated infrastructure , technically skilled manpower which are not available in most of the hospitals . With increasing incidence of dengue infections, the early diagnostic confirmation is required for timely intervention and disease control . Hence, diagnosis of dengue disease during the acute phase should be the priority and is a public health concern. In a developing country like India where resources are minimal a Rapid immunochromatographic test method could be useful as a point of care test to provide early diagnosis for timely intervention[48].

In the study 7 cases (4%) out of 176 were IgM positive by immunochromatographic test [Table7] . By ELISA method a total of 37 (21%) out of 176 cases were positive for IgM .This 37 cases included 7 cases which were IgM positive by immunochromatographic test .So all the IgM positive cases detected by Immunochromatographic test were positive by ELISA also . Additionally 30 samples were detected as IgM positive by ELISA method. Here we can see that these patients may have presented in the later stage of the primary infection by dengue virus. In addition, IgM antibody response varies considerably among individuals due to host humoral immune response. By immunochromatographic test , no samples was found to be positive by IgG but by ELISA 7 (4%) were detected as IgG positive. So we can see that these seven IgG positive patients may have presented during their secondary infection. A high level of IgG is an indicator of infection or it represents endemicity[54]. A high degree of cross reactivity is mostly observed in flavivirus infection because the flaviviruses share common group epitopes particularly on the

envelope (E) protein[44]. The dengue serological tests become more difficult, because dengue antibodies are cross reactive with other flaviviruses such as West Nile virus (WNV), Japanese encephalitis virus (JEV) and Yellow fever virus (YFV). Moorthy (2009) has evaluated a rapid immunochromatographic device for detection of IgM and IgG antibodies to dengue viruses done in tertiary care hospital . Accuracy indices for IgM and IgG detection respectively were positive predictive value of 61% and 72% and negative predictive value of 89% and 83% respectively . it was concluded that the device performs poorly in the detection of IgM and IgG antibodies to dengue virus and is not recommended for use as a stand –alone diagnostic test[80].

In this study an attempt has been made to find out the association of dengue serological marker with thrombocytopenia . Platelet count less than 1oo,ooo/ml is taken as thrombocytopenia. Out of the 176 cases 123 cases (70%) has shown thrombocytopenia .The comparison of platelet counts with dengue specific parameters is shown in table 7. Out of the 57 cases positive for NS1 antigen , thrombocytopenia was evident in 42 cases (74%). In contrast , when IgM and IgG antibodies were compared only 20 (45%) out of 44 cases had thrombocytopenia. Therefore early viraemic phase coincides with high NS1 antigen levels in the blood which in turn is related to thrombocytopenia[1].

Table13. Comparison of platelet counts with various permutations and combinations of dengue parameters.

SEROLOGICAL MARKERS	TOTAL	PLATELET COUNT LESS THAN 100,000ML	PERCENTAGE
NS1 antigen	57	42	74%
IgM antibody	37	18	48%
IgG antibody	7	2	28%

From the above table it can also be observed that , thrombocytopenia is observed in 48% of IgM antibody positive cases and 28% of the IgG positive cases. Association of thrombocytopenia with NS1 was found to be higher by statistical test (Table 1, 3) ; Multivariant Regression Analysis with dependent binary variables . The p value has been found to be less than .05 ($p < 0.05$) which is found to be highly significant. From the above results it could be concluded that thrombocytopenia is more commonly associated with NS1 antigen positive cases than NS1 negative cases. Association of thrombocytopenia with NS1was found to be 74% while association of thrombocytopenia with IgM and IgG was found to be 50%. Association of thrombocytopenia with IgM is found to be significant (p value= 0.018) whereas not much association is found between thrombocytopenia and IgG levels (p value =0.045) (Table 2, 5). The mechanism of dengue related thrombocytopenia and coagulopathy is quite complex . So viraemia correlates with NS1 positivity and thrombocytopenia. It would involve platelet activation , pro-coagulant and anticoagulant arms of the coagulation system , complement , cytokines , and

endothelial cells . Moreover symptomatic thrombocytopenia would require platelet transfusion though platelet counts might not correlate well with clinical bleeding[11] .

Saurabh (2013) studied the importance of platelet count and serological markers in diagnosing dengue infection . Of the 159 samples tested , 17 were positive for one or more dengue parameters . Of the seventeen , 05 were positive for NS1 only , 06 showed IgM only while 01 showed IgG only . Thrombocytopenia was more consistently associated whenever NS1 was detected compared to antibody detection[109] .

In the present study 75 cases of fever , in which none of the dengue parameters was positive , thrombocytopenia was noted in 22 cases . Platelets are decreased in several other conditions like some viral infections other than dengue , drug induced thrombocytopenia , collagen vascular diseases, idiopathic thrombocytopenia etc[1] .

This study is conducted in a tertiary care teaching hospital which lack a viral culture set up . Therefore applying gold standard tests in the studies related to viral infection is not practical for diagnosis and treatment purpose . The precise day of fever at the time of conducting the test could not be obtained in a large number of cases. NS1 antigen was positive in 32% of cases by both tests. It indicates that if the samples were taken on the first day of fever more number of cases would have been picked up by NS1 antigen[57] . It is shown that the titres of NS1 represents the viral load and the viral load is directly proportional to the complications. Conventional ELISA require at least 4 hours whereas rapid immunochromatographic test requires twenty minutes, which will be helpful in initiating the treatment and minimizing the complications and mortality of dengue[120]. But the lack of conformity

in the evaluvation of Dengue rapid diagnostic tests (RDTs) remains a problem and a standardised approach must be performed when performing diagnostic assessment . Moreover studies have demonstrated that Rapid diagnostic test cannot reliably differentiate the different dengue infection states (primary or secondary dengue infection). The manufacturers of the kit used in this study allows the use of serum , plasma or whole blood for use in dengue rapid diagnostic tests[1] . Unfortunately there is little evidence that all sample types perform equally well . The effect of anticoagulants and whole blood on RDT performance and ease of reading also require examination in a field setting[11] .

Summary

SUMMARY

Dengue fever is a vector borne disease which is rapidly spreading in urban areas in tropical country like India. The clinical and laboratory findings in dengue are very similar to those of other febrile disease that are prevalent in the same geographical regions, Therefore, a dengue diagnostic test is required for adequate case management.

- This study supports the concept that in comparison to IgM and IgG antibodies for dengue fever, NS1 antigen assay is an effective tool for diagnosis of dengue infection.
- NS1 antigen assay holds promise particularly in early diagnosis of dengue infection when antibodies are not at a detectable level.
- NS1 antigen assay significantly improves the diagnostic algorithm without the requirement of paired sera .
- In this study the efficacy of immunochromatographic test in the diagnosis of dengue infection is evaluvated by comparing with ELISA , an accepted standard test method for dengue diagnosis.
- The results for NS1 antigen by immunochromatographic test is comparable with that of NS1 antigen ELISA .
- In case of IgM and IgG antibody detection , the immunochromatographic test method is inferior to ELISA.
- Thrombocytopenia was more observed in NS1 antigen positive cases. Viraemia correlates with NS1 positivity and so indirectly correlated with thrombocytopenia.

Conclusion

CONCLUSION

1) NS1 antigen detection is a useful tool in the early diagnosis of dengue infection and deserves inclusion in the routine diagnostic algorithm of suspected dengue fever cases.
2) Detection of all three parameters NS1 antigen/ IgM , IgG antibodies simultaneously gives more information regarding the primary and secondary infection.
3) Rapid immunochromatographic test methods are very useful for early diagnosis of dengue infection and case management, where sophisticated infrastructure and expertise are scarce.

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CONSENT FORM

PART 1 OF 2

INFORMATION FOR PARTICIPANTS OF THE STUDY

Dear Volunteers,

We welcome you and thank you for your keen interest in participation in this research project. Before you participate in this study, it is important for you to understand why this research is being carried out. This form will provide you all the relevant details of this research. It will explain the nature, the purpose, the benefits, the risks, the discomforts, the precautions and the information about how this project will be carried out. It is important that you read and understand the contents of the form carefully. This form may contain certain scientific terms and hence, if you have any doubts or if you want more information, you are free to ask the study personnel or the contact person mentioned below before you give your consent and also at any time during the entire course of the project.

Title of the Study :

Detection of NS1 antigen / IgG IgM antibodies in early Dengue Virus infection among the patients attending Sree Mookambika Institute of Medical Science, Kulasekharam.

Name of the Investigator : **Dr. VIDHYA. V.R.**
Post Graduate Student
Dept of Microbiology
SMIMS, Kulasekharam.

Name of the guide : **Dr. N. PALANIAPPAN . M.D**
Professor, Department of Microbiology,
SMIMS, Kulasekharam.

Name of the Co-guide : **Dr. P. Indu (M.D)**
Professor and Head
Department of Microbiology,
SMIMS, Kulasekharam.

Institute : Sree Mookambika Institute of Medical Sciences (SMIMS)
Kulasekharam, Kanyakumari District, Tamilnadu.

CONSENT FORM

Introduction :

Dengue has become a major international public health problem due to human morbidity and mortality it cause. Dengue virus is an arthropod borne it causes. Dengue virus is an arthropod borne, Flavivirus that can be found in tropical and sub – tropical regions world wide. The World Health organization estimates that there may be up to 100 million dengue infection world wide which could result up to 250,000 to 500,000 cases of Dengue Haemorrhagic Fever each year (Gibbons & Vaughn, 2002, WHO, 1997).

The Fever Could Present as

- Mild Dengue fever
- Dengue hemorrhagic fever (DHF)
- Dengue shock syndrome (DSS)

Incubation period is 5-11 days. The disease presents as acute febrile illness with chills, headache, retro-oculars pain, body aches and arthralgia in more than 90% of apparent cases accompanied by nausea, vomiting and a maculopapular rash resembling measles. Lasting for 2-7 days. Illness usually persists for 7 days followed with fever remitting after 3-5 days followed by relapse (saddle – back fever) and pain in the bones muscles and joint to earn the name **Break – bone fever.**

There are 4 serotypes of Dengue designated as DEN 1 through DEN 4.

Humans are the main reservoirs for dengue virus. Transmission occurs by mosquito vectors that is well adapted to urban setting. Aedes aegypti is the primary vector mosquito for Dengue in Western hemispheres.

CONSENT FORM

NS1 protein is a 50 Kilda ton glycoprotein that is expressed on the surface of infected cells and is found in both secreted and non secreted forms. Dengue NS1 antigen has been detected in the serum of DEN virus infected patients as early as one day post onset of symptoms (DPO) Upto 18 DPO.

Procedure for the study :

You are required to participate in this study only if you fully understand and agree to the requirements for the same. There will be no difference in the treatment you receive, nor will treatment be withheld based on your decision to participate in this study. The study is done in collaboration with Department of microbiology. Hundred fever patients who volunteer are included in this study. After considering inclusion and exclusion criteria.2ml of Blood samples will be collected from the fever patients, with fever of duration less than 5 days. Blood from the OP patients will be collected from the ward and blood from the IP patient will be collected from the ward.The test Kit is SD Boiling Dengue. Duo Rapid Test Kit which detects the presence of Dengue NS1 Ag on the side and Dengue IgG/ IgM antibodies on there side. Your age, address IP No, History of illness will all be taken initially.

Expected risks for the participants : Moderate risk

Expected benefits of research for the participants :

Fever could be diagnosed as Dengue fever in its early phase itself. This may help the clinician in early diagnosis and treatment of fever cases. If may be graftifying to know that your contribution is indispensable for advancement of medical knowledge.

Confidentiality :

All your study records will be kept confidential. Your personal identity will not be revealed in any publication or release of results. Study records will be kept indefinitely for analysis and follow up.

Compensation to the participants : NIL

Can I withdraw from the study at any time during the study period?

Yes

If there is any new findings / information, would I be informed?

Yes

What happens in case of a study related injury?

Any adverse event as experienced due to the study will be treated as per hospital guidelines.

For any study related queries, you are free to contact,

Dr. Vidhya. V.R.
PG Student
Department of Microbiology
SMIMS, Kulasekharam
Mobile : 918891041013
amalsnair@gmail.com

Place : Kulasekharam **Signature of investigator**

Date :

CONSENT FORM

PART – II

The details of the study have been explained to me in writing and the details have been fully explained to me. I am aware that the results of the study may not be directly beneficial to me but will help in the advancement of medical sciences. I confirm that I have understood the study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). I have been given an information sheet giving details of the study. I fully consent to participate in the study titled Detection of NSI antigen / IgG IgM antibodies in early Dengue virus infection among the patients attending SRee Mookambika Institute of Medical Science.

Name : **Address :**

Hospital No :

Signature of the participant

Witness

1.

2.

Date :

Place : Kulasekharam

PROFORMA

Sree Mookambika Institute of Medical Science, Kulasekharam

DEPARTMENT OF MICROBILOGY

Study Title

Detection of NS1 antigen / IgG IgM antibodies in early Dengue virus infection among the patients attending Sree Mookambika Institute of Medical Science, Kulasekharam.

- Serial No : Date :

- Name :

- Age :

- Address :

- Contact Number :

- Hospital reference Numbers :

- History of fever duration :

- Previous history / arthralgia :

- Previous history of Dengue fever :

-Platelet count :